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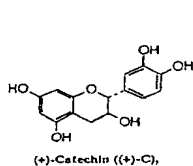
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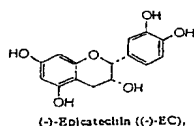
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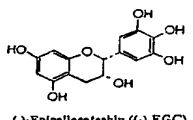
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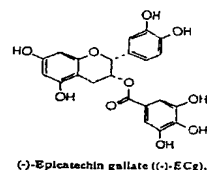
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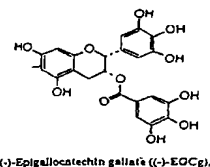
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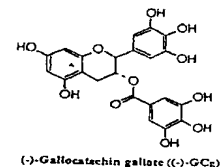
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(57) Abstract: A method of treating an amyloid disease, or a disease characterized by alpha-synuclein or NAC fibrillogenesis, in a mammalian subject. The method includes administering to the mammal a therapeutically effective amount of a various disclosed catechins or green tea extract. A pharmaceutical composition comprising a therapeutically effective amount of a catechin and pharmaceutically acceptable excipient. The therapeutic amount of the catechin or green tea extracts is selected for efficacy in treating amyloid, alpha-synuclein or NAC fibrillogenesis in a mammalian subject.

WO 03/013442 A2

WO 03/013442 A2



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Title: CATECHINS FOR THE TREATMENT OF FIBRILLOGENESIS
IN ALZHEIMER'S DISEASE, PARKINSON'S DISEASE,
SYSTEMIC AA AMYLOIDOSIS, AND OTHER AMYLOID DISORDERS

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This application is a continuation-in-part of US application serial number 09/753,313 filed 12/29/00; it also claims priority to US provisional applications 60/276,866 filed 3/15/01, and 60/338,969 filed 12/10/01.

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TECHNICAL FIELD

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The invention relates to compositions and methods for treating Alzheimer's Disease and other amyloidoses, and to methods for isolating pharmaceutical agents from plant matter; more particularly, it relates to uses, compositions and methods for therapeutic intervention in Alzheimer's disease, systemic AA amyloidosis, and other amyloid disorders, and the treatment of diseases characterized by alpha-synuclein/NAC (i.e. non-amyloid component) fibril formation, especially Lewy body disease, Parkinson's disease, and multiple system atrophy, using catechins, other plant matter and derivatives thereof.

BACKGROUND OF THE INVENTION

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Alzheimer's disease is characterized by the accumulation of a 39-43 amino acid peptide termed the beta-amyloid protein or A β , in a fibrillar form, existing as extracellular amyloid plaques and as amyloid within the walls of cerebral blood vessels. Fibrillar A β amyloid deposition in Alzheimer's disease is believed to be detrimental to the patient and eventually leads to toxicity and neuronal cell death, characteristic hallmarks of Alzheimer's disease. Accumulating evidence implicates amyloid as a major causative factor of Alzheimer's disease pathogenesis.

Parkinson's disease is another human disorder characterized by the formation, deposition, accumulation and/or persistence of abnormal fibrillar protein deposits that demonstrate many of the characteristics of amyloid. In Parkinson's disease, an accumulation of cytoplasmic Lewy bodies consisting of filaments of alpha-synuclein/
5 NAC are believed important in the pathogenesis and as therapeutic targets. New agents or compounds able to inhibit alpha-synuclein/NAC formation, deposition, accumulation and/or persistence, or disrupt pre-formed alpha-synuclein/NAC fibrils (or portions thereof) are regarded as potential therapeutics for the treatment of Parkinson's disease.

10 A variety of other human diseases also demonstrate amyloid deposition and usually involve systemic organs (i.e. organs or tissues lying outside the central nervous system), with the amyloid accumulation leading to organ dysfunction or failure. These amyloid diseases (discussed below) leading to marked amyloid accumulation in a number of different organs and tissues are known as systemic amyloidoses. In other
15 amyloid diseases, single organs may be affected such as the pancreas in 90% of patients with type 2 diabetes. In this type of amyloidosis, the beta-cells in the islets of Langerhans in pancreas are believed to be destroyed by the accumulation of fibrillar amyloid deposits consisting primarily of a protein known as islet amyloid polypeptide (IAPP). Inhibiting or reducing such amyloid accumulation is believed to lead to new
20 effective treatments for type 2 diabetes. In Alzheimer's disease, Parkinson's and "systemic" amyloid diseases, there is currently no cure or effective treatment, and the patient usually dies within 3 to 10 years from disease onset.

The amyloid diseases include, but are not limited to, the amyloid associated with Alzheimer's disease, Down's syndrome, hereditary cerebral hemorrhage with
25 amyloidosis of the Dutch type, and inclusion body myositis (Askanas et al, Ann. Neurol. 43:521-560, 1993) (wherein the specific amyloid is referred to as beta-amyloid protein or A β), the amyloid associated with chronic inflammation, various forms of malignancy and Familial Mediterranean Fever (wherein the specific amyloid is referred to as AA amyloid or inflammation-associated amyloidosis), the amyloid
30 associated with multiple myeloma and other B-cell dyscrasias (wherein the specific amyloid is referred to as AL amyloid), the amyloid associated with type II diabetes (wherein the specific amyloid protein is referred to as amylin or islet amyloid polypeptide), the amyloid associated with the prion diseases including Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, kuru and animal scrapie (wherein the

specific amyloid is referred to as PrP amyloid), the amyloid associated with long-term hemodialysis and carpal tunnel syndrome (wherein the specific amyloid is referred to as beta₂-microglobulin amyloid), the amyloid associated with senile cardiac amyloid and Familial Amyloidotic Polyneuropathy (wherein the specific amyloid is referred to as transthyretin or prealbumin), and the amyloid associated with endocrine tumors such as medullary carcinoma of the thyroid (wherein the specific amyloid is referred to as variants of procalcitonin).

In addition, the alpha-synuclein protein which forms fibrils, and is Congo red and Thioflavin S positive, is found as part of Lewy bodies in the brains of patients with Parkinson's disease, Lewy body disease (Lewy in Handbuch der Neurologie, M. Lewandowski, ed., Springer, Berlin pp.920-933, 1912; Pollanen et al, J. Neuropath. Exp. Neurol. 52:183-191, 1993; Spillantini et al, Proc. Natl. Acad. Sci. USA 95:6469-6473, 1998; Arai et al, Neurosc. Lett. 259:83-86, 1999), and multiple system atrophy (Wakabayashi et al, Acta Neuropath. 96:445-452, 1998).

Discovery and identification of new compounds or agents as potential therapeutics to arrest amyloid formation, deposition, accumulation and/or persistence that occurs in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes, and other amyloidoses are desperately sought.

DISCLOSURE OF THE INVENTION

The identification and use of standardized green tea extract and derivatives and constituents thereof, such as the catechin compounds shown for example in Figure 1, are disclosed for the therapeutic intervention of Alzheimer's disease, systemic AA amyloidosis and other amyloidoses, and Parkinson's and Lewy body diseases.

In addition, methods of isolation for the identification and purification of the potent amyloid inhibitory ingredients within green tea extract are disclosed. Use of standardized green tea leaf extract and its ingredients (i.e. 50% polyphenols) contained within different commercial preparations are anticipated to benefit human patients with Alzheimer's disease and other amyloidoses, and Parkinson's and Lewy body diseases, due to green tea leaf extract's ability to inhibit amyloid fibril formation, and Parkinson's alpha-synuclein fibril and Lewy body formation, and to cause dissolution/disruption, and disaggregation of pre-formed amyloid and alpha-synuclein fibrils.

We disclose the identification of and make the surprising discovery that commercially available catechins, such as those present in standardized green tea

extracts, and the standardized green tea leaf extract itself (such as found in extract standardized to 50% polyphenols) act as an impressive inhibitor of Alzheimer's disease amyloid fibril formation, systemic AA amyloid fibril formation, and Parkinson's disease alpha-synuclein/NAC fibril formation. Furthermore, commercially available catechins (including but not limited to epicatechin, catechin, gallic acid, epigallocatechin gallate, epigallocatechin, and/or epicatechin gallate) and standardized green tea leaf extract have the ability to cause a disassembly/disruption of pre-formed amyloid fibrils of the Alzheimer's type, suggesting that this agent may be useful for patients at latter stages Alzheimer's disease, and those affected with other amyloid diseases. Standardized green tea leaf extract and catechins obtained from different commercial sources (extracts isolated from gelatin-coated capsules) were found to serve as potent inhibitors of Alzheimer's disease amyloid fibrillogenesis and Parkinson's disease protein fibrillogenesis.

For purposes of this disclosure, Parkinson's disease, due to the fact that fibrils develop in the brains of patients with this disease (which are Congo red and Thioflavin S positive, and which contain predominant beta-pleated sheet secondary structure), is identified and discussed as a disease which also displays the characteristics of an amyloid-like disease, and disclosures and claims herein related to amyloidoses are therefore expected in like manner to relate therapeutically to Parkinson's and Lewy body diseases. Therefore agents or compounds found to inhibit Alzheimer's disease A β amyloid fibril formation, are anticipated to also be effective in the inhibition of alpha-synuclein/NAC fibril formation. These agents or compounds will therefore also serve as therapeutics for Parkinson's and Lewy body diseases, in addition to having efficacy as a therapeutic for Alzheimer's disease, systemic amyloidosis, type 2 diabetes, and other amyloid disorders.

Selected polyhydroxylated aromatic compounds, such as epicatechin, appear to be effective as inhibitors of systemic AA amyloidosis *in vivo*. Significantly, we have discovered that catechin itself (the epimer of epicatechin) is not effective as an inhibitor of systemic AA amyloidosis. We identify various polyhydroxylated aromatic compounds that inhibit fibrillar AA amyloid formation and/or deposition and that are useful for the treatment of systemic AA amyloidosis found in patients with chronic inflammatory disorders.

Both commercially available standardized green tea leaf extract and various catechins caused a marked significant dose-dependent inhibition of A β 1-40 amyloid

fibril formation as determined using a Thioflavin T fluorometry assay in a dose-dependent manner. Standardized green tea leaf extract and catechins obtained from commercial sources were also potent disrupters of pre-formed A β 1-42 containing amyloid fibrils, as determined using a Thioflavin T fluorometry assay, and exerted their effects in a dose-dependent manner. Selected polyhydroxylated aromatic compounds, such as epicatechin appear to be effective inhibitors of systemic AA amyloidosis in an experimental mouse model. Lastly, standardized green tea leaf extract obtained from different commercial sources caused a disaggregation of pre-formed A β 1-42 Alzheimer's amyloid fibrils, and catechins obtained from different commercial sources caused an inhibition of NAC-containing Parkinson's disease fibrils.

Therefore, the use of standardized green tea leaf extract and/or specific catechins (in various forms, i.e. a pill, tablet, liquid form, powder form, etc.) and derivatives thereof, from different commercial sources is disclosed for the treatment of fibrillogenesis in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, and other amyloidoses. Also disclosed are methods of isolation to identify and purify the key amyloid inhibitory ingredients within the green tea extract material. Identification of the "active" amyloid inhibitory ingredients within the green tea extracted plant materials are anticipated to lead to new drug design for anti-amyloid therapeutics of the future. Current use of standardized green tea leaf extract and its ingredients, such as catechins as contained within different commercial preparations, are anticipated to benefit human patients at all stages of Alzheimer's, Parkinson's and other amyloid or amyloid-like diseases due to catechin's and standardized green tea extract's newly demonstrated ability to inhibit A β amyloid fibril formation, and alpha-synuclein fibril formation (important for treating early to mid-stage Alzheimer's disease and Parkinson's disease, respectively), and cause dissolution/disruption and disaggregation of pre-formed amyloid fibrils (important for treating mid to late stages of Alzheimer's disease and Parkinson's disease, respectively). Similarly, catechins and standardized green tea leaf extract are anticipated to benefit patients with different systemic amyloid diseases such as systemic AA amyloidosis, and type 2 diabetes, regardless of the stage of amyloid accumulation and the organ (or tissue) involved.

While tea extract results are exemplified with Species *Camellia sinensis*, extracts from other species within the family Theaceae are believed to have similar effects.

The use of green tea, green tea leaves and extracts, and catechins or derivatives thereof, is disclosed for the treatment of amyloid and/or fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses.

5 "Treatment" is also intended in every possible instance to include and cover "*in vitro* treatment", whether for experimental or screening purposes and the like, and whether or not the *in vitro* treatment leads to, or is ever intended to lead to, treatment of a like fibrillogenesis, or any amyloid or alpha-synuclein disease corresponding to that fibrillogenesis, in a mammalian subject.

10 A method of treatment of amyloid, alpha-synuclein or NAC fibrillogenesis, in an *in vitro* environment, is also disclosed. The method includes the step of administering into the *in vitro* environment a therapeutically effective amount of a catechin, or green tea extract. Preferably the catechin is selected from the group of catechins including epicatechin, catechin, galocatechin gallate, epigallocatechin
15 gallate, epigallocatechin, and epicatechin gallate, or a derivative of one of the above compounds.

Also disclosed is the use of green tea, green tea leaves and extracts or derivatives thereof, from the *Camellia sinensis* species for the treatment of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease,
20 Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses.

Green tea, green tea leaves and extracts or derivatives thereof, from the Theaceae family are disclosed for the treatment of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic
AA amyloidosis, type 2 diabetes and other amyloidoses.

25 Commercially available pills, tablets, caplets, soft and hard gelatin capsules, lozenges, sachets, cachets, vegicaps, liquid drops, elixers, suspensions, emulsions, solutions, syrups, tea bags, tea leaves, aerosols (as a solid or in a liquid medium), suppositories, sterile injectable solutions, sterile packaged powders, and/or leaf powder which contain green tea, green tea leaves and extracts or derivatives thereof, are
30 disclosed for the treatment of patients with Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses.

Green tea, green tea leaves and extracts or derivatives thereof, and/or the polyphenols contained within green tea, green tea leaves and extracts or derivatives thereof, for the treatment of amyloid formation, deposition, accumulation and/or

persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses.

The invention is described with reference to specific embodiments, plant species and parts, methods, procedures and the like. However, it will be recognized by those skilled in the art that various chemical substitutions can be made within the disclosed compounds without departing from the spirit and scope of the invention. In particular, it is known that catechins can be isolated and/or purified from plant materials by a number of different methods. It will further be recognized that these alternate methods, and consequent changes in other steps of the method, such as use of different solvents or different columns for purification, and of catechins from a composition of partially purified polyphenols, fall within the scope of the presently disclosed plant-derived extracts, and compounds derived thereof.

Also disclosed is the use of catechins contained within green tea, green tea leaves and extracts or derivatives thereof, for the treatment of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses.

Catechins, including but not limited to, catechin, epicatechin, gallic acid, gallate, epigallocatechin gallate, epigallocatechin, and/or epicatechin gallate, whether contained within green tea, green tea leaves and extracts or derivatives thereof, or from other natural or synthetic sources, are disclosed for the treatment of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses.

Also disclosed is the use of bioflavanoids contained within green tea, green tea leaves and extracts or derivatives thereof, for the treatment of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses, and the use of flavanols contained within green tea, green tea leaves and extracts or derivatives thereof, for the treatment of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses.

Also disclosed is the use of flavandiol contained within green tea, green tea leaves and extracts or derivatives thereof, for the treatment of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses, and the use

of flavanoids contained within green tea, green tea leaves and extracts or derivatives thereof, for the treatment of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses.

5 Also disclosed is the use of tannins contained within green tea, green tea leaves and extracts or derivatives thereof, for the treatment of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses.

10 Methods to isolate the active ingredients present within green tea, green tea leaves and extracts or derivatives thereof, are also disclosed for use as potent agents which inhibit amyloid formation, amyloid deposition, amyloid accumulation, amyloid persistence, cause a disassembly/disruption and/or cause a disassembly of pre-formed or pre-deposited amyloid fibrils in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses. Methods for isolation of the
15 active ingredients within green tea, green tea leaves and extracts or derivatives thereof, include application of some standard techniques known to those skilled in the art, including, but not limited to, thin layer chromatography using silica-coated plates, and separation and isolation using high or low pressure liquid chromatography (HPLC). Other active ingredients within green tea, green tea leaves and extracts or
20 derivatives thereof, found to be potent inhibitors of amyloid formation, amyloid deposition, amyloid accumulation, amyloid persistence, and/or cause a disassembly/disruption, and disaggregation of pre-formed or pre-deposited amyloid fibrils in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses, are identified by re-testing of individual bands or
25 fractions (separated by thin layer chromatography, column chromatography and/or HPLC) using specific assay tests as described in the Examples.

Sufficient isolation of these active ingredients contained within individual bands and/or fractions are then sent out for specific analyses which may include, but are not limited to, scanning electron microscope equipped with energy dispersive x-ray
30 analyzer to detect and spatially map some elements present in each sample, elemental analysis by combustion to determine the relative % of carbon, hydrogen and nitrogen, high resolution mass spectroscopy to determine molecular weight and elemental composition, Fourier transform infrared spectroscopy to determine functional groups and make comparisons to the spectra of known compounds, differential scanning

calorimetry to determine melting point, atomic absorption, gel chromatography, high performance liquid chromatography, proton and C^{13} nuclear magnetic resonance spectroscopy for material characterization and to provide information regarding the position of atoms relative to each other, and UV/VIS spectroscopy. It is expected that additional techniques will be developed as part of the further isolation of potent active ingredients within green tea, green tea leaves and extracts or derivatives thereof.

The use of green tea, green tea leaves and extracts or derivatives thereof, and/or its ingredients [(regardless of commercial source and regardless of final form for consumption by humans, i.e. pills, tablets, caplets, soft and hard gelatin capsules, lozenges, sachets, cachets, vegicaps, liquid drops, elixers, suspensions, emulsions, solutions, syrups, tea bags, aerosols (as a solid or in a liquid medium), suppositories, sterile injectable solutions, sterile packaged powders, and/or tea leaf powder] are disclosed for inhibition of amyloid formation, deposition, accumulation, and/or persistence, regardless of its clinical setting.

Compositions and methods involving administering to a subject a therapeutic dose of green tea, green tea leaves and extracts or derivatives thereof, which inhibits amyloid deposition is also disclosed. Accordingly, disclosed compositions and methods are useful for inhibiting amyloidosis in disorders in which amyloid deposition occurs. The compounds of the invention can be used therapeutically to treat amyloidosis or can be used prophylactically in a subject susceptible to amyloidosis. Disclosed methods are based, at least in part, in directly inhibiting amyloid fibril formation, causing disassembly/disruption and/or disaggregation of pre-formed amyloid fibrils.

Pharmaceutical compositions for treating amyloidosis are also disclosed. The disclosed pharmaceutical compositions include a therapeutic compound in an amount effective to inhibit amyloid deposition and a pharmaceutically acceptable vehicle.

The use of any and all synthetic compounds that are similar to green tea, green tea leaves, extracts or derivatives thereof and/or its active ingredients, for use as potent agents which inhibit amyloid formation, amyloid deposition, amyloid accumulation, amyloid persistence, cause a disassembly/ disruption, and/or disaggregation of pre-formed or pre-deposited amyloid fibrils in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses, are also disclosed

In a particular aspect a method of isolation to purify and identify the amyloid inhibitory ingredients from green tea, green tea leaves and extracts or derivatives

thereof is disclosed. In one such method, an extract prepared from commercially obtained pills, tablets, caplets, soft and hard gelatin capsules, lozenges, sachets, cachets, vegicaps, liquid drops, elixers, suspensions, emulsions, solutions, syrups, tea bags, aerosols (as a solid or in a liquid medium), suppositories, sterile injectable solutions, sterile packaged powders, tea powder, using the method employing some or all of the following steps: a) extraction from green tea, green tea leaves and extracts or derivatives thereof regardless of form as described above using water or alcohol (i.e. methanol, ethanol or propanol, b) centrifugation at 2,500X g for 20 minutes and collection of supernatant, c) rotary evaporation to dryness for alcohol-extracted compounds and lyophilization for water-extracted compounds, d) washing dry powder obtained with 4 volumes of petroleum ether (repeated 4 times), followed by centrifugation (each time) at 2,500X g for 20 minutes, and collection of supernatants and pellets, e) air-drying of collected pellets, f) re-extraction of air-dried pellets with water and centrifugation at 2,500X g for 20 minutes, g) lyophilization of collected supernatants (referred to as water extracts), h) re-dissolving the pellets or lyophilized water-extract powder in acetonitrile/water/trifluoroacetic acid (TFA), i) injecting and separation by HPLC or low pressure chromatography, j) identifying amyloid inhibitory ingredients by testing in relevant *in vitro* and *in vivo* assays, and k) sending out for structural analysis and elemental composition, as described herein.

A composition, in the form of a dietary supplement, for providing, supporting or improving in a subject one or more of the mental or cognitive qualities selected from the group of mental or cognitive qualities consisting of nutritional support for age related cognitive or memory decline, normal brain function, cognitive ability, and concentration, wherein the composition comprises green tea, green tea leaves and extracts or derivatives thereof, is also disclosed.

A composition, in the form of a dietary supplement, for promoting, maintaining or enhancing in a subject one or more of the mental or cognitive qualities selected from the group of mental or cognitive qualities consisting of mental acuity, mental alertness, cognitive well being, normal brain function, cognitive ability, mental performance, memory concentration, mental sharpness, mental vitality, mental clarity, short term memory, normal brain function, learning, and good brain health, wherein the composition comprises green tea, green tea leaves and extracts or derivatives thereof, is disclosed.

A composition, in the form of a dietary supplement, for promoting or supporting healthy pancreatic function in a subject, by helping to promote normal insulin function, or for reducing, disrupting, dissolving, inhibiting, eliminating or preventing in a subject one or more conditions involving the pancreas selected from the group of conditions involving the pancreas consisting of amyloid fibril deposits, amyloid protein deposits, pancreas associated with amyloid fibril deposits, pancreas associated amyloid protein deposits, amyloid fibril formation and growth, and pancreas associated amyloid fibril formation and growth, wherein the composition comprises green tea, green tea leaves and extracts or derivatives thereof, is also disclosed.

Use of epicatechin or derivatives thereof, for the treatment of fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is disclosed.

Use of catechins, catechin hydrate or derivatives thereof, for the treatment of fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is disclosed.

Use of gallocatechin gallate or derivatives thereof, for the treatment of fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is also disclosed.

Use of epicatechin gallate or derivatives thereof, for the treatment of fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is disclosed.

Use of epigallocatechin or derivatives thereof, for the treatment of fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is disclosed.

Use of epigallocatechin gallate or derivatives thereof, for the treatment of fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is also disclosed.

Use of green tea, green tea leaves and extracts or derivatives thereof, for the treatment of fibril formation, deposition, accumulation and/or persistence in

Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is disclosed.

5 Use of green tea, green tea leaves and extracts or derivatives thereof, from the *Camellia sinensis* species for the treatment of fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is also disclosed.

10 Use of green tea, green tea leaves and extracts or derivatives thereof, from the Theaceae family for the treatment of fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is disclosed.

15 Use of commercially available pills, tablets, caplets, soft and hard gelatin capsules, lozenges, sachets, cachets, vegicaps, liquid drops, elixers, suspensions, emulsions, solutions, syrups, tea bags, tea leaves, aerosols (as a solid or in a liquid medium), suppositories, sterile injectable solutions, sterile packaged powders, and/or leaf powder which contain green tea, green tea leaves and extracts or derivatives thereof, or catechins, for the treatment of fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is also disclosed.

20 Use of catechins, including but not limited to catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate and galocatechin gallate contained within green tea, green tea leaves and extracts or derivatives thereof, for the treatment of fibril formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is also disclosed.

25 Compositions and methods involving administering to a subject a therapeutic dose of catechins or derivatives thereof, which inhibits amyloid deposition is also disclosed. Accordingly, disclosed compositions and methods are useful for inhibiting amyloidosis in disorders in which amyloid deposition occurs. The compounds can be used therapeutically to treat amyloidosis or can be used prophylactically in a subject susceptible to amyloidosis. The methods are based, at least in part, in directly inhibiting amyloid fibril formation, causing disassembly/disruption and/or disaggregation of pre-formed amyloid fibrils.

30 Use of any and all synthetic catechins or derivatives thereof and/or their active ingredients, for use as potent agents which inhibit amyloid formation, amyloid

deposition, amyloid accumulation, amyloid persistence, cause a disassembly/disruption, and/or disaggregation of pre-formed or pre-deposited fibrils in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses is disclosed.

5 A pharmaceutical agent is disclosed for treating an amyloid disease in a patient, wherein the pharmacological agent comprises a therapeutically effective amount of plant matter from a plant of the family Theraceae, and in particular the genus *Camellia*. The pharmacological agent is preferably from a plant of the genus *Camellia*, species *sinensis*. The pharmacological agent is preferably an extract
10 obtained from *Camellia sinensis*, the extract being from the dried leaves.

The pharmacological agent preferably has a therapeutically effective amount of standardized green tea leaf extract, or specific catechins, in a dosage in the range of from about 0.1 to about 500 mg/kg of body weight of the patient, and more preferably in the range from about 1.0 to about 100 mg/kg of body weight of the
15 patient.

Preferred pharmaceutical agents have a weight percentage of plant extract in the agent is in the range of from about 70% to about 95%, and may also have a pharmaceutically acceptable carrier, diluent or excipient. The pharmaceutical agent preferably has an amyloid inhibitory activity or efficacy greater than 50%.

20 Disclosed compositions also have the ability to reduce, eliminate, prevent, inhibit, disrupt, disassemble, or disaggregate amyloid fibril or protein deposits, brain associated amyloid fibril deposits or brain associated amyloid protein deposits, as well as amyloid fibril formation, or age associated amyloid fibril formation, brain associated amyloid fibril formation; it will therefore promote mental acuity, promote mental
25 alertness, provide nutritional support for age or related cognitive or memory decline, promote cognitive well being, support brain function, improve cognitive ability, mental performance or memory, promote concentration and mental sharpness, improve mental vitality, promote greater mental clarity and alertness, improve short term memory, reduce or reverse age associated cognitive or memory decline, support normal
30 brain function, enhance learning or memory; improve concentration, enhance mental performance, reduce mental decline, reduce likelihood of age related brain disorders, and maintain good brain health.

It is anticipated that disclosed compositions also have the ability to reduce, eliminate, prevent, inhibit, disrupt, disassemble or disaggregate amyloid fibril or

protein deposits, pancreas associated amyloid fibril or protein deposits, as well as amyloid fibril formation and growth, and pancreas associated amyloid fibril formation and growth; it will therefore support healthy pancreatic function and promote pancreatic function by helping to promote normal insulin function.

5 Disclosed compositions may also include carriers, diluents and/or excipients commonly used in the pharmaceutical and dietary supplement industries and any such additions as will be known to those skilled in the art are acceptable and may be employed without departing from the scope of the invention.

10 A method for isolating amyloid inhibitory constituents within green tea, green tea leaves, extracts or derivatives thereof is also disclosed, the method comprising the following steps:

a) extracting the green tea, green tea leaves, extracts or derivatives thereof with water, or with an organic solvent, b) removing insoluble materials, c) evaporation to dryness or lyophilization to obtain powder, d) recovering and redissolving the amyloid inhibitory constituents obtained in the water or organic solvent, and e) 15 injecting and separation by high pressure or low pressure liquid chromatography.

Representative constituents of green tea include catechins, bioflavanoids, flavanols, flavandiols, flavanoids, tannins or derivatives thereof, although for purposes of this disclosure these substance may optionally also be synthetically derived or 20 independently found in other plant sources.

The step of extracting the plant matter with an organic solvent further comprises adding methanol initially to plant materials that are powdered, and the resulting mixture is stirred overnight. The solvent used in the step of extracting amyloid inhibitory ingredients preferably has a polarity ranging from that of water to 25 that of pentanol. The step of removing insoluble materials is preferably affected by centrifuging the extract and collecting the supernatant. The step of concentrating the extract is preferably effected by rotary evaporation or lyophilization (for water extracts). Following the extraction and centrifugation steps, the extraction and centrifugation procedure is preferably repeated 1 to 5 more times and the 30 supernatants are collected.

Following the repeated steps of extraction and concentration, the supernatants are preferably pooled and dried using a rotary evaporator or lyophilization (for water extracts). The dry powder is washed with 4 volumes of petroleum ether (repeated 4 times), followed by centrifugation (each time) at 2,500X g for 20 minutes, and

collection of the supernatants and pellets. The collected pellets are air-dried and re-extracted with water and centrifuged at 2,500X g for 20 minutes. The collected supernatants (referred to as water extracts) are lyophilized, and the pellets or lyophilized water-extract powder are re-dissolved in acetonitrile/water/trifluoroacetic acid (TFA) for HPLC injection or low pressure chromatography. The dissolved pellet is divided into equal portions and injected into an HPLC. The HPLC preferably contains a 1X25 cm C₁₈ column, though other sizes may be made to serve, and is maintained at 30°C with a flow rate of 2 ml/min. The sample portions injected onto the HPLC are eluted with gradients of A and B, such that 0% B for 5 minutes, 0-15% B from 5-10 minutes, 15-45% B from 10-70 minutes, and 45-100% B from 70-85 minutes; where B=95% acetonitrile with 0.5% acetic acid in distilled water and A=5% acetonitrile with 0.5% acetic acid in distilled water. The eluents from the HPLC are monitored at all wavelengths and 4 ml fractions are collected in a fraction collector and pooled peaks are obtained at various retention times (from 0 through 85 minutes). The fractions obtained may be concentrated by lyophilization after most of the acetonitrile is removed by rotary evaporation.

The concentrated fractions obtained are then tested in relevant *in vitro* assays to identify potent inhibitors of amyloid fibril formation, or disassembly/disruption or disaggregation of pre-formed amyloid fibrils. The amyloid inhibitory ingredients are preferably drawn from the HPLC approximate retention times of 10-70 minutes.

A method is also disclosed for treating an amyloid disease in a patient comprising the step of administering to the patient a therapeutically effective amount of green tea, green tea leaves and extracts or derivatives thereof. The green tea, green tea leaves, and extracts or derivatives thereof, are preferably administered orally or by aerosol spray or in a parentally injectable or infusible form.

In disclosed methods, amyloid formation, deposition, accumulation and/or persistence in a subject is inhibited by administering a therapeutic dose of the invention to the subject. The term "subject" is intended to include living organisms in which amyloidosis can occur. Examples of subjects include humans, monkeys, cows, sheep, goats, dogs, cats, mice, rats and transgenic species thereof. Administration of green tea, green tea leaves and extracts or derivatives thereof, to a subject to be treated can be carried out using known procedures, at dosages and for periods of time effective to inhibit amyloid formation, deposition, accumulation and persistence in the subject. An effective amount of the therapeutic compound necessary to achieve a

therapeutic effect may vary according to factors such as the amount of amyloid already deposited at the clinical site in the subject, the age, sex, and weight of the subject, and the ability of the therapeutic compound to inhibit amyloidosis in the subject.

Representative Method or Process Embodiments

5 A method of treatment, prevention, or management of an amyloidosis in a mammalian subject susceptible to, or afflicted by, the amyloidosis is presented, the method comprising the step of administering to the subject a therapeutic amount of plant matter from a source of green tea, green tea leaves, standardized green tea extract, or green tea derivative.

10 A method for the treatment, inhibition, prevention or management of amyloid formation, deposition, accumulation, aggregation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses in a mammalian subject is presented, the method comprising the step of administering to the subject a therapeutic amount of a substance selected from the
15 group of substances consisting of green tea, green tea leaves, standardized green tea extract, green tea derivative, catechins, bioflavanoids, flavanols, flavandiols, flavanoids, tannins or derivatives thereof.

 The substance is preferably a catechin selected from the group of catechins consisting of catechin, epicatechin, gallic acid, gallic acid gallate, epigallocatechin gallate,
20 epigallocatechin, and epicatechin gallate, or a derivative of one of the above group.

 In any of these embodiments, within the step of administering plant matter, a therapeutic quantity of one or more plant materials selected from the group of plants consisting of, and commonly known as, Cat's claw, ginkgo biloba, rosemary, gotu kola, bacopin, and ginseng may also optionally be administered.

25 A method for the treatment, inhibition, prevention or management of alpha-synuclein fibril formation, deposition, accumulation, aggregation and/or persistence in Parkinson's disease, Lewy body disease, or multiple system atrophy, in a mammalian subject is presented, the method comprising the step of administering to the subject a therapeutic amount of a substance selected from the group of substances
30 consisting of green tea, green tea leaves, standardized green tea extract, green tea derivative, catechins, bioflavanoids, flavanols, flavandiols, flavanoids, tannins or derivatives thereof.

The substance is preferably a catechin selected from the group of catechins consisting of catechin, epicatechin, gallic catechin gallate, epigallocatechin gallate, epigallocatechin, and epicatechin gallate, or a derivative of one of the above group.

5 A method for promoting mental alertness in a patient is presented, the method comprising the step of administering to the patient a therapeutically effective amount of plant matter from a plant of the family Theaceae, and preferably from a plant of the genus *Camellia*, species *sinensis*. This method may also be used for inhibiting the formation of brain amyloid deposits.

10 A method for promoting, maintaining or enhancing in a patient one or more of the mental or cognitive qualities selected from the group of mental or cognitive qualities consisting of mental acuity, mental alertness, cognitive well being, normal brain function, cognitive ability, mental performance, memory, concentration, mental sharpness, mental clarity, short term memory, normal brain function, and learning is presented, the method comprising the step of administering to the patient a
15 therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

A method for providing, supporting or improving in a patient one or more of the mental or cognitive qualities selected from the group of mental or cognitive qualities consisting of normal brain function, cognitive ability, and concentration is presented,
20 the method comprising the step of administering to the patient a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

A method for reducing in a patient one or more of the mental or cognitive effects selected from the group of mental or cognitive effects consisting of, age associated cognitive or memory decline, mental decline, and likelihood of age related brain or
25 cognitive disorders is presented, the method comprising the step of administering to the patient a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

A method for reducing, disrupting, dissolving, inhibiting, eliminating or preventing in a patient one or more conditions involving the brain selected from the
30 group of conditions involving the brain consisting of amyloid fibril deposits, amyloid protein deposits, brain associated amyloid fibril deposits, brain associated amyloid protein deposits, amyloid fibril formation and growth, age associated amyloid fibril formation and growth, brain associated amyloid fibril formation and growth is presented, the method comprising the step of administering to the patient a

therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

A method for promoting or supporting healthy pancreatic function in a patient, by helping to promote normal insulin function is presented, the method comprising the
5 step of administering to the patient a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

A method for reducing, disrupting, dissolving, inhibiting, eliminating or preventing in a patient one or more conditions involving the pancreas selected from the group of conditions involving the pancreas consisting of amyloid fibril deposits,
10 amyloid protein deposits, pancreas associated amyloid fibril deposits, pancreas associated amyloid protein deposits, amyloid fibril formation and growth, pancreas associated amyloid fibril formation and growth is presented, the method comprising the step of administering to the patient a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

15 Representative Use And/or Composition/Agent Embodiments

The use of a source of green tea, green tea leaves or standardized green tea leaf extract or derivatives thereof in the preparation of a pharmaceutical composition or dietary supplement for the treatment, prevention and or management of an amyloidosis in a mammalian subject susceptible to, or afflicted by, the amyloidosis is
20 presented.

The use of a source of green tea, green tea leaves, standardized green tea leaf extract or derivatives thereof in the preparation of a pharmaceutical composition or dietary supplement for inhibiting amyloid fibril formation, deposition, accumulation, or persistence or causing dissolution/disruption or disaggregation of pre-formed
25 amyloid fibrils is presented.

A pharmaceutical composition or dietary supplement for the treatment, prevention, or management of amyloidosis in a mammalian subject susceptible to, or afflicted by, the amyloidosis is presented, the composition comprising a source of green tea, green tea leaves or standardized green tea leaf extract or derivatives thereof, and,
30 if desired, a pharmaceutically or dietarily acceptable carrier, diluent or excipient.

A pharmaceutical composition or dietary supplement for inhibiting amyloid fibril formation, deposition, accumulation, or persistence or causing dissolution/disruption and or disaggregation of pre-formed amyloid fibrils is presented, the composition comprising a source of green tea, green tea leaves or standardized green

tea leaf extract or derivatives thereof and, if desired, a pharmaceutically or dietarily acceptable carrier, diluent or excipient.

5 The use of catechins, bioflavanoids, flavanols, flavandiols, flavanoids, tannins or derivatives thereof for the treatment, prevention or management of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses, in a mammalian subject susceptible to the amyloidosis is presented.

10 A pharmaceutical composition or dietary supplement for the treatment, prevention or management of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses in a mammalian subject susceptible to such amyloid condition is presented, the composition comprising catechins, bioflavanoids, flavanols, flavandiols, flavanoids, tannins or derivatives thereof and, if desired, a pharmaceutically or dietarily acceptable carrier, diluent or excipient.

15 A pharmaceutical composition or dietary supplement for the treatment, inhibition, prevention or management of alpha-synuclein fibril formation, deposition, accumulation, aggregation and/or persistence in Parkinson's disease, Lewy body disease, or multiple system atrophy in a mammalian subject is presented, the composition comprising a substance selected from the group of substances consisting
20 of green tea, green tea leaves, standardized green tea extract, green tea derivative, catechins, bioflavanoids, flavanols, flavandiols, flavanoids, tannins or derivatives thereof.

The use of a source of green tea, green tea leaves or standardized green tea leaf extract or derivatives thereof in the preparation of a pharmaceutical composition or
25 dietary supplement for providing, supporting or improving in a subject one or more of the mental or cognitive qualities is presented.

The use of a source of green tea, green tea leaves or standardized green tea leaf extract or derivatives thereof in the preparation of a pharmaceutical composition or dietary supplement for promoting or supporting healthy pancreatic function in a
30 subject is presented.

A pharmaceutical composition or dietary supplement for providing, supporting or improving in a subject one or more of the mental or cognitive qualities which comprises a source of green tea, green tea leaves or standardized green tea leaf extract or derivatives thereof is presented.

A pharmaceutical composition or dietary supplement for promoting or supporting healthy pancreatic function in a subject which comprises a source of green tea, green tea leaves or standardized green tea leaf extract or derivatives thereof is presented.

5 A pharmacological agent for promoting, maintaining or enhancing in a patient one or more of the mental or cognitive qualities selected from the group of mental or cognitive qualities consisting of mental acuity, mental alertness, cognitive well being, normal brain function, cognitive ability, mental performance, memory, concentration, mental sharpness, mental vitality, mental clarity, short-term memory, normal brain
10 function, and learning, and good brain health is presented, wherein the pharmacological agent comprises a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

A pharmacological agent for providing, supporting or improving in a patient one or more of the mental or cognitive qualities selected from the group of mental or
15 cognitive qualities consisting of nutritional support for age related cognitive or memory decline, normal brain function, cognitive ability, and concentration is presented, wherein the pharmacological agent comprises a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

A pharmacological agent for reducing in a patient one or more of the mental or
20 cognitive effects selected from the group of mental or cognitive effects consisting of, age associated cognitive or memory decline, mental decline, and likelihood of age related brain or cognitive disorders is presented, wherein the pharmacological agent comprises a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

25 A pharmacological agent for reducing, disrupting, dissolving, inhibiting or preventing in a patient one or more conditions involving the brain selected from the group of conditions involving the brain consisting of amyloid fibril deposits, amyloid protein deposits, brain associated amyloid fibril deposits, A β brain deposits, brain associated A β deposits, brain associated amyloid protein deposits, brain amyloid
30 deposits, amyloid fibril formation and growth, age associated amyloid fibril formation and growth is presented, wherein the pharmacological agent comprises a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

A pharmacological agent for promoting or supporting healthy pancreatic function in a patient, by helping to promote normal insulin function is presented, wherein the pharmacological agent comprises a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

5 A pharmacological agent for reducing, disrupting, dissolving, inhibiting or eliminating or preventing in a patient one or more conditions involving the pancreas selected from the group of conditions involving the pancreas consisting of amyloid fibril deposits, amyloid protein deposits, pancreas associated amyloid fibril deposits, amylin deposits, islet amyloid polypeptide deposits, pancreas associated amyloid protein
10 deposits, amyloid fibril formation and growth, pancreas associated amyloid fibril formation and growth is presented, wherein the pharmacological agents comprises a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

For a more detailed discussion of and background information pertaining to
15 amyloid and amyloidosis, Alzheimer's disease and the aging population, amyloid as a therapeutic target for Alzheimer's disease and Parkinson's disease and alpha-synuclein fibril formation, see our co-pending patent application 10/077,596 and issued patents 6,037,327, 6,264,994 and 6,346,280, which discussion and background is incorporated herein by this reference as if fully set forth.

20 These and other features and advantages will become more fully apparent when the following detailed description is read in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a set of structures for catechin derivatives.

25 FIGURE 2 is a black and white graph of a Thioflavin T fluorometry assay utilized to determine the dose-dependent effects of standardized green tea leaf extract on inhibition of Alzheimer's A β 1-40 amyloid fibril formation.

FIGURE 3 is a black and white graph of a Thioflavin T fluorometry assay utilized to determine the dose-dependent effects of standardized green tea leaf extract on
30 disassembly/disruption of pre-formed Alzheimer's A β 1-42 amyloid fibrils.

FIGURE 4 is a black and white graph of a Congo red-A β spectrophotometric assay to determine the effects of standardized green tea leaf extract (from 2 commercial sources) on disaggregation of pre-formed Alzheimer's A β 1-42 fibrils.

FIGURE 5 is a black and white graph of a Thioflavin T fluorometry assay utilized to determine the dose-dependent effects of different commercially available catechins on inhibition of Alzheimer's A β 1-40 amyloid fibril formation.

FIGURE 6 is a color photomicrograph demonstrating the inhibition of Alzheimer's A β 1-40 fibril formation by epicatechin.

FIGURE 7 is a black and white graph of a Thioflavin T fluorometry assay utilized to determine the dose-dependent effects of commercially available catechins on disassembly/disruption of pre-formed Alzheimer's A β 1-42 amyloid fibrils.

FIGURE 8 is a black and white graph of a Thioflavin T fluorometry assay utilized to determine the dose-dependent effects of different commercially available catechins on inhibition of Parkinson's disease NAC (also referred to as "NAC-P") fibril formation.

FIGURE 9 shows black and white photomicrographs of amyloid deposition in spleen in a mouse model of experimental AA amyloidosis.

FIGURE 10 shows black and white photomicrographs of amyloid deposition in liver in a mouse model of experimental AA amyloidosis.

FIGURE 11 is a graph demonstrating the effect of specific polyphenolic compounds on disruption/disassembly of pre-formed amyloid fibrils consisting of islet amyloid polypeptide (IAPP).

FIGURE 12 is a graph of circular dichroism spectroscopy demonstrating the disruption of β -sheet structure in A β 1-42 amyloid fibrils by epicatechin.

FIGURE 13 is a graph demonstrating the inhibition of splenic amyloid by epicatechin, but not catechin hydrate, in an experimental mouse model of systemic AA amyloidosis.

FIGURE 14 shows black and white photomicrographs of inhibition of splenic AA amyloid deposition by epicatechin in a mouse model of systemic AA amyloidosis.

FIGURE 15 illustrates the structures of some of the polyhydroxylated aromatic containing-compounds conceived to have anti-amyloid activity.

BEST MODE OF CARRYING OUT THE INVENTION

Turning now to the Drawings and Examples, the invention will be described in preferred embodiments by detailed reference to them.

Some Definitions

"Pharmaceutically acceptable analogs and derivatives." Pharmaceutically acceptable analogs and derivatives of a claimed compound include various R- group

type substitutions, and any other derivative structural modifications not affecting the disclosed efficacy of these compounds.

“Percentage Purity.” Disclosed compositions contain one or more catechins or green tea derivatives, each catechin or green tea derivative present in the composition in a proportion percentage or percentage purity that “significantly exceeds” a proportion percentage of the same substance’s natural presence in a plant, or in an extract from the plant. For example, suppose that a particular catechin is present in a plant in a percentage by weight of 0.01 percent, and is present in an extract of the plant in a percentage by weight of 1.0 percent. In a disclosed composition then, the same catechin is present in the composition in a percentage by weight that is significantly greater than 0.01 percent or 1.0 percent, say 10 percent. Other proportionalities along this line may be applied as well, such as percentage composition or percentage presence by volume, or percent purity.

By way of further example, without limiting the scope of invention to this or other examples, a catechin is present in a tablet to be delivered orally in accordance with the disclosure herein. The catechin is an isolated catechin present in a percentage purity of 98.5% (that is, the catechin is 98.5% pure, as measured by conventional purity indicia, such as for example the characteristic single sharp peak band on an HPLC). The particular catechin is however only a 15% ingredient by weight in the tablet. The catechin is known to be present in a plant in a dry weight percentage of 0.06, while certain extracts of the same plant are known to contain up to 0.75 percent dry weight percent of the same catechin. In this example, the catechin is proportionally more present in the tablet than in the extract by a ratio of 20:1, and this is one measure of significantly exceeding the natural proportion percentage presence in a plant or extract of the plant.

In general, a catechin present in a therapeutically administered dose form that has a percentage of the catechin (by weight, dry weight, volume, or purity) that is 10 times (or more) greater than the natural percentage presence of the same catechin in a plant is a percentage that “significantly exceeds” the natural percentage presence of the catechin in the plant. When speaking of extracts of a plant in this context, it should be noted that only conventional or natural extracts are to be considered (juices, concentrates and the like, or extracts known and used for other purposes), not new extracts prepared after the priority date of this disclosure the effect of which is to concentrate the particular catechin so as to negative a finding of “significantly

exceeding", as just defined. It should also be noted that in some cases, a finding of "significantly exceeding" may be justified with a ratio of as little as 2:1, but more preferably as great as 50:1 to 100:1.

5 In the example of a single catechin compound with an excipient to make up the composition, it may be convenient merely to note and compare the percent purity of the compound in the composition, rather than its overall weight percentage, for purposes of the "significantly exceeding" standard, as claimed. In the case of mixtures of catechins it can be appropriate to view a combined percentage composition of the mixed catechins in the therapeutic dosage and compare that figure to a combined
10 percentage presence of the same catechin's in the natural plant or extract.

In any event, the purpose of the disclosed standard of measurement is to set forth a fair margin by which a claimed composition exceeds reading on the active ingredients' natural occurrence in plants and conventional extracts of plants.

Preferred compositions will contain catechin that is at least substantially pure.
15 Catechin that is in substantially pure isolated or synthetic form may be advantageously employed as well. In general "pure" means better than 95% pure, and "substantially pure" catechin means a catechin purified by extraction or other known means or means disclosed herein such that the catechin is present in the therapeutic dosage with only those impurities that can not readily nor reasonably be removed by
20 the extraction or purification processes. "Isolated" means that the catechin in question is not accompanied in the therapeutic form by significant quantities of other catechins. An "isolated pure" compound is a compound in isolated purified form such as is conventional for active ingredients in the pharmaceutical industry.

Islet Amyloid Polypeptide (IAPP) and Type 2 Diabetes

25 Islet amyloid deposits are observed in ~90% of patients with well-established type 2 diabetes and would appear to be a characteristic feature of the disease process (Westermarck, J. Med. Sci. 77:91-94, 1972; Clark et al, Diabetes Res. 9:151-159, 1988). In many patients the deposits are widespread and affect many islets. The degree of islet (predominantly β -cell) mass that has been replaced by amyloid may be a marker
30 for the severity of the diabetic disease process, with those individuals requiring insulin treatment having the greatest islet mass reduction and amyloid formation (Westermarck, Amyloid: Int. J. Exp. Clin. Invest. 1:47-60, 1994). Since islet amyloid has been observed in autopsy samples obtained from different populations, it appears to be a phenomenon common to the disease rather than to a subpopulation of individuals

with the syndrome (Westermarck, J. Med. Sci. 77:91-94, 1972; Clark et al, Diabetes Res. 9:151-159, 1988). The prevalence of islet amyloid deposits increases with age (Bell, Am. J. Path. 35:801-805, 1959), which is not surprising because normal aging is associated with a deterioration in glucose tolerance and an increased prevalence of type 2 diabetes (Davidson, Metabolism 28:687-705, 1979).

The major protein in islet amyloid is a 37-amino acid peptide known as islet amyloid polypeptide (IAPP) or amylin. IAPP is a known normal secretory product of the pancreatic β -cells (Kanh et al, Diabetes 39:634-638, 1990) that is stored in insulin-bearing cytoplasmic granules (Clark et al, Cell Tissue Res. 257:179-185, 1989). It has long been questioned whether the deposition of islet amyloid is involved in or merely a consequence of the pathogenesis of type 2 diabetes. However, a number of studies now suggest that in fact islet amyloid formation, deposition and persistence may be an important primary factor leading to β -cell dysfunction and cell death, hyperglycemia, and in the development of type 2 diabetes.

IAPP has been hypothesized to have an important role in the pathogenesis of type 2 diabetes through its impairment of β -cell function and reduction of β -cell mass (Johnson et al, N. Engl. J. Med. 321:513-518, 1989). Besides being able to form islet amyloid deposits that replace β -cell mass, amyloid fibrils appear to damage islets directly. Studies in which islets were incubated in the presence of human or rat IAPP demonstrated that human IAPP formed amyloid fibrils in a concentration-dependent manner and was associated with the death of pancreatic islet β -cells (Lorenzo et al, Nature 368:756-760, 1994). Cell death did not occur in the presence of rat IAPP that does not form amyloid fibrils (Lorenzo et al, Nature 368:756-760, 1994).

Studies involving transgenic mouse models have allowed further insight into the role of islet amyloid in the pathogenesis of type 2 diabetes. More recent studies do suggest that development of IAPP-derived islet amyloid does not depend on hyperglycemia and is progressive (Verchere et al, Proc. Natl. Acad. Sci. U.S.A. 93:3492-3496, 1996). In these latter studies hyperglycemia developed in only 31% of male transgenic mice, and in 14% of male nontransgenic animals. When pancreatic sections from these mice were examined, islet amyloid was found in every transgenic mouse with diabetes. However, two-thirds of male transgenic animals that were normoglycemic also developed islet amyloid deposits indicating that hyperglycemia was not a prerequisite for islet amyloid formation. The data from these and other studies further suggested that human IAPP fibrils may be cytotoxic to β -cells and thus

could produce early alterations in islet function (Lorenzo et al, Nature 368:756-760, 1994; Janson et al, Diabetes 47:A250, 1998). Islet amyloid deposition appears to be an early feature of the islet lesion of type 2 diabetes and progressive accumulation of islet amyloid is associated with further β -cell mass reduction (Clark et al, Diabetes Res. 5 9:151-159, 1988; Westermark and Wilander, Diabetologia 15:417-421, 1978). Thus, a progressive reduction in islet mass caused by increased amyloid deposition is associated with a progressive impairment in insulin secretion, reduction in glucose tolerance, and eventually the development of fasting hyperglycemia. The studies in transgenic animals suggest not just that hyperglycemia is associated with the development of islet amyloid, but that amyloid contributes to the development of hyperglycemia by replacing β -cells. These studies as a whole suggest that islet amyloid formation plays a central role in the development of β -cell failure of type 2 diabetes. Therefore, agents or compounds able to inhibit or disrupt islet amyloid (i.e. IAPP or amylin) formation, deposition, accumulation or persistence may lead to new potential treatments for type 2 diabetes.

Systemic AA Amyloidosis

Systemic AA amyloidosis is characterized by the deposition and accumulation of insoluble fibrillar amyloid deposits consisting of the AA amyloid protein, into a number of different organs including heart, kidney, liver, spleen, gastrointestinal tract, lungs, and skin. Such amyloid accumulation and persistence can be fatal especially if deposition includes the heart (leading to heart failure) or kidneys (leading to kidney failure). Systemic AA amyloidosis is generally associated with chronic inflammatory disorders and is observed in patients with rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, osteomyelitis, leprosy, tuberculosis, Hodgkin's disease, renal cell carcinoma, and a familial form of disease known as Familial Mediterranean Fever. Currently, there is no cure or effective treatment for systemic AA amyloidosis and the patient usually dies within 3-7 years from disease onset. It is estimated that in the US there are approximately 50,000 - 100,000 cases of systemic AA amyloidosis, whereas in Europe the number of cases are estimated to be 2-5 fold higher.

Systemic AA amyloidosis involves a particular amyloid protein (referred to as the "AA amyloid" protein) that is deposited in a fibrillar form and is generally associated with chronic inflammatory diseases including rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel diseases, osteomyelitis, chronic infections

(e.g. tuberculosis), Hodgkin's disease, leprosy, renal cell carcinoma, and in a familial form known as Familial Mediterranean Fever (McAdam et al, Lancet 2:572-573, 1975; Benson and Cohen, Arth. Rheum. 22:36-42, 1979; Metaxis, Kidney Int. 20:676-685, 1981; Kamei et al, Acta Path. Jpn. 32:123-133, 1982). Amyloid deposition in these diseases may occur in a variety of different organs including heart, kidney, liver, spleen, gastrointestinal tract, skin and/or lungs (Kisilevsky, Can. J. Physiol. Pharmacol. 65:1805-1815, 1987). The most common feature of systemic AA amyloidosis is proteinuria, with or without a reduction in the glomerular filtration function of the kidneys. Such amyloid deposition in the kidney may lead to renal failure, whereas amyloid deposition in the heart may lead to heart failure. Thus, the consequences of AA amyloid deposition in systemic organs may be fatal to the patient. Currently, a majority of patients with diagnosed AA amyloidosis, generally have a high mortality rate, within 3-7 years from disease onset (Kisilevsky, Can. J. Physiol. Pharmacol. 65:1805-1815, 1987; Cohen and Connors, J. Path. 151:1-10, 1987). Currently there is no cure or effective treatment, and the discovery of new potential therapeutics for the treatment of AA amyloidosis is desperately needed.

Exact numbers of the cases of systemic AA amyloidosis have not been well documented. However, systemic AA amyloidosis have been reported to occur in approximately 2-5% of rheumatoid arthritic patients in the United States, and in about 5-10% of cases in Europe (Fillipowicz-Sosnowska et al, Arth. Rheum. 21:699-703, 1978; Pras et al, John Hopkins Med. J. 150:22-26, 1982; David et al, Clin. Exp. Rheum. 11:85-90, 1993). This disease also occurs in 5-17% of cases of juvenile rheumatoid arthritis in Europe, 1% of cases of Crohn's disease in the United States and Europe, and 15-27% of patients with Familial Mediterranean Fever in the United States and Europe, respectively (Fillipowicz-Sosnowska et al, Arth. Rheum. 21:699-703, 1978; Mataxas, Kidney Int. 20:676-685, 1981; David et al, Clin. Exp. Rheum. 11:85-90, 1993; Saatci et al, Acta Ped. 81:705-706, 1993). It is estimated that there are about 50,000 - 100,000 cases of systemic AA amyloidosis cases in the United States (Hazenberg and Van Rijswijk, In: Reactive Amyloidosis and the Acute Phase Response, edited by G. Husby, Baillere's Clinical Rheumatology, 8:661-690, 1994), with a potential market size of approximately \$20-\$40 million per year in the US alone. In Europe, the number of cases are estimated to be approximately 100,000-400,000.

AA Amyloid and SAA

AA amyloid is common to a host of seemingly unrelated disorders. As described above, clinically AA amyloidosis can be a complication of long-standing inflammation, familial disorders, and various forms of malignancy. A common protein being deposited in such different clinical settings suggests that there is a common pathogenetic mechanism operative in all cases (Kisilevsky, Can. J. Physiol. Pharmacol. 65:1805-1815, 1987).

The AA amyloid protein consists of 76 amino acids with a molecular weight of ~8,500 (Ein et al, J. Biol. Chem. 247:5653-5655, 1972; Sletten et al, Biochem. Biophys. Res. Comm. 69:19-25, 1976; Erikson et al, Proc. Natl. Acad. Sci. 73:964-967, 1976).

These 76 amino acids correspond to the amino terminal two thirds of a naturally occurring serum protein that is known as SAA or apoSAA (Hoffman et al, J. Exp. Med. 159:641-646, 1984). ApoSAA is an acute phase protein whose serum concentration increases rapidly (within 24 hours) and substantially (500-2000 fold) during any inflammatory response (McAdam and Sipe, J. Exp. Med. 144:1121-1127, 1976; McAdam et al, J. Exp. Med. 144:1121-1127, 1978; Sipe, Br. J. Exp. Path. 59:305-310, 1978). The amino acid sequence of the AA amyloid protein is identical from patient to patient, regardless of the underlying nature of the inflammatory disturbance that has preceded its deposition. The sequence is also identical to the AA amyloid protein seen in Familial Mediterranean Fever and in patients with Hodgkin's disease or renal cell carcinoma. The AA protein has been found in a number of different species, including humans, ducks, mink, mice and monkeys (Sletten et al, Biochem. Biophys. Res. Comm. 69:19-25, 1976; Gorevic et al, J. Immunol. 121:138-140, 1977; McAdam et al, J. Clin. Invest. 61:390-394, 1978; McAdam and Sipe, J. Exp. Med. 144:1121-1127, 1978; Sipe, Br. J. Exp. Path. 59:305-310, 1978; Waalen et al, Eur. J. Biochem. 104:407-412, 1980). Aside from amino acid substitutions that have occurred during evolution, the AA protein is essentially the same to that seen in human (Westermarck et al, Comp. Biochem. Physiol. Comp. Biochem. 85:609-614, 1986).

In its native state SAA exists as a 160,000 to 200,000 molecular weight complex associated with high-density lipoprotein (Benditt et al, Proc. Natl. Acad. Sci. U.S.A. 74:4025-4028, 1977; Skogen et al, Scand. J. Immunol. 6:1363-1368, 1977; 1979; Benditt et al, Proc. Natl. Acad. Sci. U.S.A. 76:4092-4096, 1979; Marhaug et al, Clin. Exp. Immunol. 50:382-389, 1982; Carlson and Holmquist, Lancet 1:192-197, 1983). This association likely occurs within the serum following the secretion of SAA (Hoffman et al, J. Biol. Chem. 257:10510-10517, 1982; J. Biol. Chem. 257:10518-10522, 1982). SAA

can, however, be denatured to release a 12,000 to 14,000 molecular weight subunit, of approximately 103 amino acids, designated SAAL, and all of the immunologic cross-reactivity that exists between SAA and AA proteins are found within the SAAL subunit (Benditt, Proc. Natl. Acad. Sci. U.S.A. 76:4092-4096, 1979; Skogen et al, J. Immunol. 6:1363-1368, 1977). Polymorphisms of SAAL has been demonstrated in both animals (Gorevic et al, J. Immunol. 121:138-140, 1978) and man (Marhaug et al, Clin. Exp. Immunol. 45:97-106, 1981; Parmelee et al, Biochem. 21:3298-3303, 1982).

SAA is believed to be mainly synthesized by the liver (Selinger et al, Nature 285:498-500, 1980; Sztein et al, J. Immunol. 129:87-90, 1982; Tatsuta et al, Ann. N.Y. Acad. Sci. 389:467-475, 1982) under the induction by the monokine, interleukin-1 (Sztein et al, Cell Immunol. 63:164-176, 1981). During an inflammatory reaction there is a marked increase in the mRNA in liver coding for SAA synthesis such that 2-5% of hepatic protein synthesis is devoted to this protein (Morrow et al, Proc. Natl. Acad. Sci. U.S.A. 78:4718-4722, 1981). SAA has also been reported to be present in a variety of tissue and cells which include the spleen (Watanabe et al, Am. J. Clin. Path. 67:540-544, 1977; Sipe et al, Br. J. Exp. Path. 59:305-310, 1978) connective tissue (Linder et al, J. Exp. Med. 144:1336-13346, 1976), fibroblasts (Linder et al, J. Exp. Med. 146:1158-1163, 1977); B cells following stimulation (Rosenstreich et al, Infect. Immunol. 23:181-183, 1979), and in polymorphonuclear leukocytes (Rosenthal et al, J. Clin. Invest. 62:1181-1186, 1978).

The mRNA for apoSAA has been isolated and cDNA probes prepared (Morrow et al, Proc. Natl. Acad. Sci. U.S.A. 78:4718-4722, 1981). These studies have demonstrated that there is more than one gene for apoSAA (Meek and Benditt, J. Exp. Med. 164:2006-2017, 1986; Lowell et al, J. Biol. Chem. 261:8442-8452, 1986). Within this family at least two functional genes have been identified in both human and mouse, and of these only the product of one, apoSAA₂, is deposited as the AA protein in tissues (Meek et al, J. Exp. Med. 164:2006-2017, 1986). Minor differences in amino acid sequence exist between apoSAA₁ and apoSAA₂, yet these are sufficient to lead to altered metabolic processing of one of the peptides.

Production of the AA amyloid protein in various tissues is clearly a cellular function. At present, the main cellular candidate for this function is the macrophage or the reticuloendothelial cell. ApoSAA cleaved to AA-sized polypeptides was demonstrated *in vitro* utilizing monocytes (Lavie et al, J. Exp. Med. 148:1020-1031, 1978; J. Immunol. 125:175-180, 1980), polymorphonuclear leukocytes (Silverman

et al, Immunol. 46:737-744, 1982), serum-associated serine proteases (Skogen et al, Scand. J. Immunol. 11:643-648, 1980) and macrophages (Shirahama et al, Lab. Invest. 62:61-68, 1990; Palm et al, APMIS 105:603-608, 1997; Kluve-Beckerman et al, Am. J. Path. 155:123-133, 1999).

5 A Cell Culture Method to Generate AA Amyloid

A number of previous studies indicate that AA amyloid formation can be achieved in cell culture model systems (Shirahama et al, Lab. Invest. 62:61-68, 1990; Palm et al, APMIS 105:603-608, 1997; Kluve-Beckerman et al, Am. J. Path. 155:123-133, 1999). Probably, the best cell culture system in which to produce fibrillar AA amyloid protein was described by Kluve-Beckerman et al (Am. J. Path. 155:123-133, 1999). In this *in vitro* system, murine peritoneal macrophage cells (isolated from the peritoneal cavity of normal C57BL/6 mice) were maintained, to which recombinant SAA2 (rSAA2) in neutral pH medium, and/or amyloid enhancing factor (AEF)(discussed below) were added. The medium containing recombinant SAA2 was used at a concentration typical to that seen in acute phase serum (i.e. 140µg/ml). Within 24-48 hours after treatment, cultures developed foci of AA amyloid deposits, which enlarged and became increasingly congophilic and birefringent (following Congo red staining as viewed under polarized light). This study demonstrated that SAA2 was cleaved by the macrophages to form AA amyloid protein (~8.5 kDa fragment) in culture, which was fibrillar (as demonstrated by positive Congo red birefringence and Thioflavin S fluorescence). The peritoneal cells were found to be viable (as determined by exclusion of trypan blue staining) and maintained phagocytosis activity (as shown by the phagocytosis of latex beads). In addition, cultures of peritoneal cells obtained from nude mice demonstrated similar amyloid formation and deposition in culture, indicating that T lymphocytes were not essential for amyloid fibril formation.

Although AEF was not required, its addition to the culture medium resulted in larger and more numerous amyloid deposits. A greater amount of fibrillar AA amyloid deposition in culture was also observed when cultures were treated with pepstatin, an aspartic protease inhibitor (Kluve-Beckerman et al, Am. J. Path. 155:123-133, 1999). These cultures also maintained viability similar to those of untreated cultures, even as long as 24 days. Pepstatin-treated cultures developed large areas of dense, localized masses of amyloid associated with cell clusters.

This cell culture system is both easy to employ and amenable to manipulation for the development of AA amyloid deposition and persistence *in vitro*.

A Relevant Mouse Model of Experimental AA Amyloidosis

A number of good experimental animal models exist for the production of systemic AA amyloidosis *in vivo*. Early studies involving repeated injections of azocasein (derivative of casein) demonstrated the accumulation of AA amyloid deposits in spleen, liver and kidney occurred within 14-21 days following treatment (Janigan and Druet, Am. J. Path. 48:1013-1024, 1966). In this model, the injection of the azocasein resulted in a sterile abscess, which caused an inflammatory reaction inducing elevated serum levels of SAA in the mouse (Kindy and DeBeer, Methods Enzym. 309:701-716, 1999). The AA amyloid deposits were fibrillar as indicated by their positive staining with Congo red (i.e. red/green birefringence as viewed under polarized light).

In more recent animal models, systemic AA amyloid deposition can be induced rapidly in CBA/J mice following a single subcutaneous injection of 2% silver nitrate (the inflammatory stimulus) plus a single intravenous injection of amyloid enhancing factor (AEF)(discussed below). In this model, AA amyloid deposition occurs first in the spleen (by 24-48 hours), then in liver (by 3-4 days), followed by kidney (5-7 days)(Axelrad et al, Am. J. Path. 78:277-284, 1975; Kisilevsky et al, Lab. Invest. 37:544-553, 1977). We have previously utilized this model extensively to study the development of AA amyloidosis and the role that specific proteoglycans/glycosaminoglycans play in amyloid formation (Kisilevsky et al, Appl. Path. 2:308-315, 1984; Snow et al, Lab. Invest. 53:37-44, 1985; Lab. Invest. 56:665-675, 1987; Lab. Invest. 57:687-698, 1987; J. Histochem. Cytochem. 39:1321-1330, 1991). This relevant experimental model is already in use and is specifically utilized to identify and test the efficacy of potential AA amyloid therapeutics, as described below.

The experimental CBA/J mouse model (as described above) is an ideal system to test the efficacy of potential AA amyloid therapeutics since following an inflammatory stimulus (i.e. silver nitrate) and AEF, 100% of test animals will deposit amyloid in tissues within the span of a few days. Whereas only a small percentage of patients with precursor proteins actually develop amyloid, all animals in this experimental model do so. The inflammatory reaction in CBA/J mice, unlike rheumatoid arthritis or osteomyelitis, for example, results in the appearance of all the prerequisites for amyloid deposition to occur.

AEF dramatically shortens the time necessary for the experimental induction of AA amyloid deposition in CBA/J mice and can be demonstrated in tissues 24 - 48

hours before the appearance of amyloid (Axelrad et al, Lab. Invest. 47:139-146, 1982). Although the actual identity of AEF has not been fully resolved, it is believed to be generally protein in nature (Axelrad et al, Lab. Invest. 47:139-146, 1982), or consists of a small nidus of amyloid fibril-like material that contains abundant β -sheet (Kisilevsky et al, Amyloid: Int. J. Exp. Clin. Invest. 6:98-106, 1999). AEF is usually prepared from amyloid-laden spleens and/or livers of animals previously induced for AA amyloid accumulation following daily azocasein injections or single injections of AEF and silver nitrate. These amyloidotic tissues are homogenized in phosphate-buffered saline (PBS) containing protease inhibitors, and the pellet is re-homogenized a number of times. The second through fourth supernatants (obtained following water extractions) generally contain potent AEF activity that is used to rapidly induce AA deposition in animals.

Discovery and identification of new compounds or agents as potential therapeutic agents to arrest amyloid fibrillar amyloid formation, deposition, accumulation and/or persistence that occurs in Alzheimer's disease, systemic AA amyloidosis, type 2 diabetes, and other amyloidoses, and fibrillogenesis in Lewy body disease, Parkinson's disease, and multiple system atrophy, are desperately sought. Use of Catechins, Bioflavanoids, Flavanols, Flavandiols, Flavanoids, Tannins (or derivatives of any of these) from Green Tea, Green Tea Leaves, Green Tea Extracts or Green Tea Derivatives, or from other Natural or Synthetic Sources

Green tea, and other natural sources such as black tea and wine, are known to contain catechins, bioflavanoids, flavanols, flavandiols, flavanoids, tannins or derivatives thereof. See Sugita-Konishi et al, "Epigallocatechin gallate and gallic acid in green tea catechins inhibit extracellular release of Vero toxin from enterohemorrhagic E. coli", Biochemica et Biophysica Acta, 1472, 42-50 (1999), and Fernandez et al, "HPLC determination of catechins and caffeine in tea", Analyst 125: 421-425 (2000), the texts of which are hereby incorporated by reference as if fully set forth. These constituents are now believed by us to play a significant role in the surprisingly beneficial effects of green tea in amyloidoses as discussed below.

In particular the catechins of the group consisting of catechin, epicatechin, gallic acid, epigallocatechin gallate, epigallocatechin, and/or epicatechin gallate or a derivative of one of the above group, are now believed by us to be effective, either alone or in combination with other amyloid inhibitory ingredients such any plant matter from the group of plants consisting of, and commonly known as, Cat's

claw, ginkgo biloba, rosemary, gotu kola, bacopin, and ginseng, to achieve any or all of the beneficial effects described below.

General structures for representative catechins are presented in Figure 1. It is expected that pharmaceutically acceptable analogs and derivatives of these catechin structures, such as for example various R- group type substitutions, and other derivative structural modifications not affecting the disclosed efficacy of these compounds may be made without affecting the scope of the appended claims.

Use of Standardized Green Tea Leaf Extract to Inhibit Amyloidosis

The Examples illustrated below all serve well to establish that, at least *in vitro*, green tea, green tea leaves and extracts or derivatives thereof, have the ability to inhibit the formation of brain amyloid deposits that occur during normal aging and in a variety of brain disorders including Alzheimer's disease. In addition, it is known that patients who accumulate brain amyloid deposits eventually lose cognitive ability and memory function and sustain a marked reduction in mental clarity in general. Therefore it follows that inhibition of such brain amyloid deposits will at the least promote mental alertness in such patients.

The Examples also establish that again, at least *in vitro*, green tea, green tea leaves and extracts or derivatives thereof, have the ability to reduce, eliminate, prevent, inhibit, disrupt/dissolve, or disaggregate amyloid fibril or protein deposits, as well as amyloid fibril formation, or age associated amyloid fibril formation, and brain associated amyloid fibril formation. In addition, it is known that patients who accumulate amyloid fibril or protein deposits, brain associated fibril deposits or brain associated amyloid protein deposits, or who display symptoms of amyloid fibril formation and growth or age associated amyloid fibril formation and growth, brain associated amyloid fibril formation and growth, in general will eventually lose mental acuity, mental alertness, concentration, cognitive well being, or some measure of brain function or cognitive ability, mental performance or memory, or concentration and mental sharpness, or mental vitality, or mental clarity and alertness, short term memory, or some of the ability to learn and remember. It is also known that such patients are subject to age associated or related cognitive or memory decline, or will sustain a marked reduction in mental clarity.

It follows then that inhibition, reduction, elimination, prevention, disruption, disassembly or disaggregation of such amyloid fibril or protein deposits, brain associated amyloid fibril deposits or brain associated amyloid protein deposits, or

amyloid fibril formation and growth, or age associated amyloid fibril formation and growth, will improve mental acuity, promote mental alertness, provide nutritional support for age related cognitive or memory decline, promote cognitive well being, support brain function, improve cognitive ability, mental performance or memory, promote concentration and mental sharpness, improve mental vitality, promote greater mental clarity and alertness, improve short term memory, reduce or reverse age associated cognitive or memory decline, support normal brain function, enhance learning or memory; improve concentration, enhance mental performance, reduce mental decline, reduce likelihood of age related brain disorders, and maintain good brain health, in such patients.

The Examples further suggest that green tea, green tea leaves and extracts or derivatives thereof, should have the ability to reduce, eliminate, prevent, inhibit, disrupt, dissolve, disassemble, disaggregate amyloid fibril or protein deposits, pancreas associated amyloid fibril or protein deposits, as well as amyloid fibril formation and growth, and pancreas associated amyloid fibril formation and growth. In addition, it is known that patients who accumulate amyloid fibril or protein deposits, pancreas associated amyloid fibril or protein deposits, or who display symptoms of amyloid fibril formation and growth, pancreas associated amyloid fibril formation and growth, in general lose healthy pancreatic function or sustain a reduction in normal insulin function, leading to loss or reduction of pancreatic function. It there follows that inhibition, reduction, elimination, prevention, disruption, disassembly, dissolution or disaggregation of such amyloid fibril or protein deposits, pancreas associated amyloid fibril or protein deposits, or amyloid fibril formation and growth, pancreas associated amyloid fibril formation and growth, will support healthy pancreatic function and promote pancreatic function by helping to promote normal insulin function in such patients.

Recent studies, published after the priority date of this application, now further support these experimental results. See Hasegawa, "Preventive effect of Japanese green tea against cognitive impairment in the elderly", posters 42 and 755, proceedings of World Alzheimer Congress 2000, in *Neurobiology of Aging*, 21:18 (2000), reporting statistically significant relationship between increased drinking of green tea and higher cognitive levels.

EXAMPLES

The following examples are put forth so as to provide those with ordinary skill in the art with the disclosure and description and use of commercially available green tea extract and catechins which surprisingly are shown to cause an inhibition, disassembly/disruption and/or disaggregation of Alzheimer's disease A β -containing fibrils, and Parkinson's disease NAC fibrils. However, it should not be construed that the invention is limited to these specific examples.

Example 1

Standardized Green Tea Leaf Extract is a Potent Inhibitor of Alzheimer's A β (1-40) Amyloid Fibril Formation

A previously described method of measuring amyloid fibril formation utilizing Thioflavin T fluorometry (H Naiki et al, Lab. Invest. 65:104-110, 1991; H Levine III, Protein Sci. 2:404-410, 1993; H Levine III, Amyloid: Int. J. Exp. Clin. Invest. 2:1-6, 1995; H Naiki and K. Nakakuki, Lab. Invest. 74:374-383, 1996) was employed initially to identify whether standardized green tea leaf extract was capable of inhibiting Alzheimer's A β amyloid fibril formation. Using this sensitive assay, any decreases or increases in fluorescence was previously shown to correlate with a decrease or increase in the amount of amyloid fibrils (H Naiki et al, Lab. Invest. 65:104-110, 1991; H Levine III, Protein Sci. 2:404-410, 1993; H Levine III, Amyloid: Int. J. Exp. Clin. Invest. 2:1-6, 1995; H Naiki and K. Nakakuki, Lab. Invest. 74:374-383, 1996), allowing one to determine the effects of potential inhibitors and/or enhancers of amyloid fibril formation.

In a first study, the effects of standardized green tea extract as a potent Alzheimer's disease amyloid inhibitory agent on Alzheimer's A β (1-40) fibril formation was assessed by Thioflavin T fluorometry. Thioflavin T is known to bind to fibrillar amyloid proteins, and an increase in fluorescence correlates with an increase in amyloid fibril formation, whereas a decrease in fluorescence correlates with a decrease in amyloid fibril formation. The Alzheimer's A β protein (1-40) when incubated at 37°C tends to spontaneously form amyloid fibrils that increase in quantity over time. In this study, we tested for standardized green tea extract to inhibit the Alzheimer's amyloid A β protein from forming fibrils over a 1 week period. For this study, 25 μ M of A β (1-40)(Bachem Inc., Torrance, CA, USA; Lot #WM365) was incubated in microcentrifuge tubes at 37°C for 1 week (in triplicate), either alone, or in the presence of 10 μ g/ml, 50 μ g/ml or 100 μ g/ml of standardized green tea extract in 150 mM Tris HCl, 10 mM

NaCl, pH 7.0 (TBS). For this study, the powder within one gelatin capsule of standardized green tea extract obtained from a commercial source (Nature's Resource, Mission Hills, CA) was extracted in 1 ml of distilled water and pelleted using a microcentrifuge (for 10 minutes at 2,500X g). The supernatant was then taken and lyophilized. A 1 mg/ml working solution for use in the *in vitro* assays described below was then made using distilled water. The commercial green tea leaf extracts are usually standardized to 50% polyphenols.

To assess the effects of standardized green tea extract on A β (1-40) fibril formation, 50 μ l aliquots were taken from each tube for analysis at 1 hr, 1 day, 3 days, and 1 week. For each determination described above, following each incubation period, 50 μ l of A β +/- standardized green tea extract were added to 1.2ml of 100 μ M Thioflavin T (Sigma Chemical Co., St. Louis, MO) in 50mM NaPO₄ (pH 6.0). Studies indicated that increasing concentrations of fibrillar A β gave a proportional increase in fluorescence in the presence of 100 μ M Thioflavin T, ruling out the presence of any disproportionate inner filter effects in these studies. Fluorescence emission at 482 nm was measured on a Turner instrument-model 450 fluorometer at an excitation wavelength of 450 nm. For each determination, the fluorometer was calibrated by zeroing in the presence of the Thioflavin T reagent alone, and by setting the 50 ng/ml riboflavin (Sigma Chemical Co., St. Louis, Mo) in the Thioflavin T reagent to 1800 fluorescence units. All fluorescence determinations were based on these references and any fluorescence given off by any of the compounds in the presence of the Thioflavin T reagent was always subtracted from all pertinent readings.

For all fibrillogenesis studies utilizing Thioflavin T fluorometry, as disclosed herein, comparisons of amyloid protein in the presence or absence of standardized green tea extract were based on paired Student's *t* tests with data shown as mean +/- standard deviation. Significance was reported at the 95% ($p < 0.05$), 99% ($p < 0.01$) and 99.5% ($p < 0.005$) confidence levels.

As shown in Figure 2, the effects standardized green tea extract on Alzheimer's A β (1-40) amyloid fibril formation was evaluated over a 1-week incubation period. Freshly suspended A β (1-40) alone, following a 1-hour incubation at 37°C, demonstrated an initial fluorescence of 183 +/- 10 fluorescence units. During the 1-week incubation period, there was a gradual increase in the fluorescence of A β (1-40) alone, increasing approximately 4-fold from 1 hour to 1 day, with a peak fluorescence of 852 +/- 3 fluorescence units observed at 1 day (Figure 2), consistent with previous

studies (Castillo et al, *J. Neurochem.* 69:2452-2465, 1997). Standardized green tea extract significantly inhibited A β (1-40) amyloid fibril formation in a dose-dependent manner as early as 1 hour of incubation (Figure 2). Significant inhibition ($p < 0.005$) by standardized green tea leaf extract on A β 1-40 amyloid fibril formation was observed at all time points including 1 hour, 1 day, 3 days and 1 week (Figure 2). At 1 hour, 10 μ g/ml, 50 μ g/ml and 100 μ g/ml of standardized green tea leaf extract significantly ($p < 0.005$) inhibited A β 1-40 fibril formation by 50.3 \pm 3.3%, 66.1 \pm 3.3%, and 95.1 \pm 1.6%, respectively. At 1 day, 10 μ g/ml, 50 μ g/ml and 100 μ g/ml of standardized green tea leaf extract significantly ($p < 0.005$) inhibited A β 1-40 fibril formation by 36.7 \pm 2.0%, 90.7 \pm 1.0%, and 95.9 \pm 2.0%, respectively. By 1 week, 10 μ g/ml, 50 μ g/ml and 100 μ g/ml of standardized green tea leaf extract significantly ($p < 0.005$) inhibited A β 1-40 fibril formation by 58.9 \pm 10.7%, 83.0 \pm 1.8%, and 89.3 \pm 2.1%, respectively. This initial data indicated that standardized green tea extract and at least one of its catechin, bioflavanoid, flavanol, flavandiol, flavanoid or tannin active constituents was a potent inhibitor of Alzheimer's amyloid fibril formation.

Example 2

Disassembly/Disruption of Alzheimer's Disease A β 1-42 Amyloid Fibrils by Standardized Green Tea Leaf Extract

In the next study, standardized green tea leaf extract was tested for its ability to cause a disassembly/disruption of pre-formed Alzheimer's disease amyloid fibrils containing A β 1-42. This type of activity would be important for any potential anti-amyloid compound which can be used in patients who already have substantial amyloid deposition in organs and/or tissues. For example, Alzheimer's disease patients in mid-to-late stage disease have abundant A β -containing amyloid deposits in their brains as part of both neuritic plaques and cerebrovascular amyloid deposits. A compound capable of causing disassembly/disruption of pre-existing amyloid deposits would be advantageous for use in these patients who are at latter stages of the disease process.

For this study, 1 mg of A β 1-42 (Bachem Inc., Torrance, CA, USA; Lot #516817) was dissolved in 1.0 ml of double distilled water (1 mg/ml solution). 25 μ M of A β 1-42 was then incubated overnight (~18 hours) at 37°C, in the absence or presence of 10 μ g/ml, 100 μ g/ml or 200 μ g/ml of standardized green tea leaf extract in the presence of 150 mM Tris HCl, 10 mM NaCl (pH 7.0) with 0.02% sodium azide. In these studies

(see results described below and in Figure 3), the A β 1-42:green tea extract weight ratio was 1:0.1, 1:1 and 1:2, respectively.

For this study, the powder within one gelatin capsule of standardized green tea extract obtained from a commercial source (Nature's Resource, Mission Hills, CA) was extracted in 1 ml of distilled water and pelleted using a microcentrifuge (for 10mins at 2,500X g). The supernatant was then taken and lyophilized. A 1 mg/ml working solution for use in the *in vitro* assays described below was then made using distilled water. The commercial green tea leaf extracts are usually standardized to 50% polyphenols.

A previously described method of measuring amyloid fibril formation utilizing Thioflavin T fluorometry (H Naiki et al, Lab. Invest. 65:104-110, 1991; H Levine III, Protein Sci. 2:404-410, 1993; H Levine III, Amyloid: Int. J. Exp. Clin. Invest. 2:1-6, 1995; H Naiki and K. Nakakuki, Lab. Invest. 74:374-383, 1996) was employed to assess whether standardized green tea leaf extract is capable of causing a disassembly/disruption of Alzheimer's A β 1-42 amyloid fibrils. Thioflavin T is known to bind to fibrillar amyloid proteins, and an increase in fluorescence correlates with an increase in amyloid fibril formation, whereas a decrease in fluorescence correlates with a decrease in amyloid fibrils due to disassembly and/or disruption. The Alzheimer's A β protein (1-42) when placed in solution, such as distilled water, tends to spontaneously form amyloid fibrils. Using this sensitive assay, any decreases or increases in fluorescence was previously shown to correlate with a decrease or increase in the amount of amyloid fibrils (H Naiki et al, Lab. Invest. 65:104-110, 1991; H Levine III, Protein Sci. 2:404-410, 1993; H Levine III, Amyloid: Int. J. Exp. Clin. Invest. 2:1-6, 1995; H Naiki and K. Nakakuki, Lab. Invest. 74:374-383, 1996), allowing one to identify, and quantitate the extent of potential inhibitors and/or enhancers of Alzheimer's A β 1-42 amyloid fibrils.

To assess the effects of standardized green tea extract on potential disassembly/disruption of preformed A β 1-42 fibrils, 50 μ l of A β 1-42 +/- standardized green tea leaf extract at various concentrations (described above) were added to 1.2ml of 100 μ M Thioflavin T (Sigma Chemical Co., St. Louis, MO) in 50mM NaPO₄ (pH 6.0) for fluorometry readings (as described in Example 1).

As shown in Figure 3, increasing amounts of standardized green tea extract caused a dose-dependent disassembly/disruption of pre-formed Alzheimer's A β 1-42 fibrils. A β 1-42 alone demonstrated a mean fluorescence of 780 +/- 50 fluorescence

units (Figure 3). Standardized green tea extract at 10µg/ml significantly ($p < 0.05$) caused a disassembly/disruption of Aβ 1-42 fibrils by 17±7%. On the other hand, 100µg/ml and 200µg/ml significantly ($p < 0.005$) caused a disassembly/disruption of Aβ 1-42 fibrils by 76±1.0% and 85±4.0%, respectively. This study demonstrated that standardized green tea extract and at least one of its catechin, bioflavanoid, flavanol, flavandiol, flavanoid or tannin active constituents caused disassembly/disruption of pre-formed Aβ 1-42 amyloid fibrils and was effective in a dose-dependent manner.

Example 3

Disaggregation of Alzheimer's Disease Aβ 1-42 Fibrils by Standardized Green Tea Leaf Extract

In the next study a Congo red-Aβ spectrophotometric assay (Klunk et al, Anal. Biochem. 266:66-76, 1999) was modified to determine the effectiveness of standardized green tea leaf extract on disaggregation of Alzheimer's Aβ 1-42 amyloid fibrils. For this assay, 25µM of Aβ 1-42 (Bachem Inc., Torrance CA, Lot #516817) was incubated in triplicate for 4 days in distilled water at 37°C in the absence or presence of 400µg/ml of standardized green tea extract (obtained from two commercial sources) in Tris-buffered saline (TBS)(100 mM Tris; 50 mM NaCl; pH 7.0, with 0.02% sodium azide). The Aβ: green tea extract weight ratio was 1:4. Source 1 of the standardized green tea extract used in this study was from Sundown Herbals (manufactured and distributed for Sundown Vitamins, Boca Raton, FL), whereas source 2 of the standardized green tea extract used in this study was from Nature's Resource (Mission Hills, CA). The green tea used in this study were extracted in distilled water as described in Examples 1 and 2.

Following incubation of Aβ 1-42 in the presence or absence of standardized green tea extracts (as described above)(standardized green tea extracts from the 2 sources are referred to as test compounds), 50µl of 360 µM of Congo red (Sigma Chemical Co. St. Louis, MO, USA) in distilled water was then added to 250 µl of each incubation mixture, giving a final Aβ:Congo red molar ratio of 1:3. After 10 minutes, the absorbance at 405 nm (reference wavelength to account for the absorbance of Congo red alone at 540nm) and 540 nm (sample absorbance where "sample" refers to Aβ alone, test compound alone, or Aβ + test compound, all in the presence of Congo red) was determined using a Biorad Model 550 ELISA Plate Reader (Biorad, Hercules, CA, USA). The absorbance at wavelength 405 nm was automatically subtracted by the ELISA plate reader from the absorbance at wavelength 540 nm (difference is referred

to as Δ absorbance)(Klunk et al, Anal. Biochem. 266:66-76, 1999). Therefore, the Δ absorbance reading at 540nm was proportional to the amount of aggregated A β left in solution (Klunk et al, Anal. Biochem. 266:66-76, 1999).

For all experiments involving test compounds, the Δ absorbance reading at 540nm of the test compound alone (in the absence of A β), was always subtracted from the corresponding Δ absorbance reading at 540nm of the test compound in the presence of A β .

Using this modification of the method of Klunk et al (Anal. Biochem. 266:66-76, 1999), the use of a greater final concentration of Congo red (i.e. 60 μ M instead of 14 μ M)(Anal. Biochem. 266:66-76, 1999), in the presence of fibrillar A β gave an overall absorbance at 540 nm that was always below 1.0 Absorbance Unit (AU), and well within the linear absorbance range.

Standardized green tea leaf extracts from two commercial sources were tested using the above described Congo red-A β spectrophotometric assay to determine their effectiveness on disaggregation of A β 1-42 Alzheimer's amyloid fibrils.

As shown in Figure 4, both commercially available standardized green tea extracts caused a disaggregation of pre-aggregated A β 1-42 amyloid fibrils as determined using the Congo red spectrophotometric assay described above. A β 1-40 alone demonstrated a mean Δ absorbance of 0.141 \pm 0.013 AU (Figure 4). Standardized green tea extract from source 1 (Sundown Herbals) caused a significant ($p < 0.005$) 52.5 \pm 8.5% disaggregation of 25 μ M A β 1-42 fibrils when incubated at a A β 1-42:green tea extract weight ratio of 1:4 (Figure 4). On the other hand, standardized green tea extract from source 2 (Nature's Resource) caused a significant ($p < 0.005$) 63.8 \pm 4.2% disaggregation of 25 μ M A β 1-42 fibrils when incubated at a A β 1-42:green tea extract weight ratio of 1:4 (Figure 4). Thus, independent of source, standardized green tea leaf extract and at least one of its catechin, bioflavanoid, flavanol, flavandiol, flavanoid or tannin active constituents was a potent disaggregator of Alzheimer's A β 1-42 amyloid fibrils.

Example 4

Catechins are Potent Inhibitors of Alzheimer's A β (1-40) Amyloid Fibril Formation

A previously described method of measuring amyloid fibril formation utilizing Thioflavin T fluorometry (H Naiki et al, *Lab. Invest.* 65:104-110, 1991; H Levine III, *Protein Sci.* 2:404-410, 1993; H Levine III, *Amyloid: Int. J. Exp. Clin. Invest.* 2:1-6,

1995; H Naiki and K. Nakakuki, *Lab. Invest.* 74:374-383, 1996) was employed initially to identify whether standardized green tea leaf extract was capable of inhibiting Alzheimer's A β amyloid fibril formation.

5 In a first study, the effects of commercially available catechins as a potent Alzheimer's disease amyloid inhibitory agent on Alzheimer's A β (1-40) fibril formation was assessed by Thioflavin T fluorometry. The Alzheimer's A β protein (1-40) when incubated at 37°C tends to spontaneously form amyloid fibrils which increase in quantity over time. In this study, we tested for catechins to inhibit the Alzheimer's amyloid A β protein from forming fibrils over a 1 week period. For this study, 125 μ M
10 of A β (1-40)(Bachem Inc., Torrance, CA, USA) was incubated in microcentrifuge tubes at 37°C for 1 week (in triplicate), either alone, or in the presence of catechins from different commercial sources including Epicatechin (ICN Pharmaceuticals Inc., Costa Mesa, CA), Epicatechin (Aldrich, St. Louis, MO), Catechin hydrate (Fluka Chemical-Biochemika, Ronkonkoma, New York) and Catechin (Aldrich, St. Louis, MO) in 50 mM
15 Tris HCl, 50 mM NaCl, pH 7.0 (TBS). Increasing amounts of catechins (as described above) were tested including A β :catechin at a 1:1, 1:0.1, 1:0.01 and 1:0.001 weight/weight ratio to determine if catechins exerted a dose-dependent inhibition of A β 1-40 fibril formation.

To assess the effects of catechins on A β (1-40) fibril formation, aliquots were
20 taken from each tube for analysis at 0, 3 days, and 7 days. For each determination described above, following each incubation period, 10 μ l of A β +/- catechins were added to 1.2ml of 100 μ M Thioflavin T (Sigma Chemical Co., St. Louis, MO) in 50mM NaPO₄ (pH 6.0). Studies indicated that increasing concentrations of fibrillar A β gave a proportional increase in fluorescence in the presence of 100 μ M Thioflavin T, ruling out
25 the presence of any disproportionate inner filter effects in these studies. Fluorescence emission at 482 nm was measured on a Turner instrument-model 450 fluorometer at an excitation wavelength of 450 nm. For each determination, the fluorometer was calibrated by zeroing in the presence of the Thioflavin T reagent alone, and by setting the 50 ng/ml riboflavin (Sigma Chemical Co., St. Louis, Mo) in the Thioflavin T reagent
30 to 1800 fluorescence units. All fluorescence determinations were based on these references and any fluorescence given off by any of the compounds in the presence of the Thioflavin T reagent was always subtracted from all readings.

For all fibrillogenesis studies utilizing Thioflavin T fluorometry, as disclosed herein, comparisons of amyloid protein in the presence or absence of catechins were

based on paired Student's *t* tests with data shown as mean +/- standard deviation. Significance was reported at the 95% ($p < 0.05$), 99% ($p < 0.01$) and 99.5% ($p < 0.005$) confidence levels.

The effects of commercially available catechins on Alzheimer's A β (1-40) amyloid fibril formation was evaluated over a 1-week incubation period. During the 1-week incubation period, there was a gradual increase in the fluorescence of A β (1-40) alone, with a peak fluorescence of 450 +/- 46 fluorescence units observed at 7 days (Figure 5), consistent with previous studies (Castillo et al, *J. Neurochem.* 69:2452-2465, 1997). Epicatechin (from ICN and Aldrich), catechin hydrate, and catechin all inhibited A β (1-40) amyloid fibril formation in a dose-dependent manner, and significantly ($p < 0.01$) at A β :catechin weight ratios of 1:1 and 1:0.1 (Figure 5). A 1:1 A β :epicatechin (ICN) weight/weight ratio inhibited A β fibril formation by 85%, whereas a 1:0.1 A β :epicatechin (ICN) weight/weight ratio inhibited A β fibril formation by 67%. A 1:1 A β :epicatechin (Aldrich) weight/weight ratio inhibited A β fibril formation by 86%, whereas a 1:0.1 A β :epicatechin (ICN) weight/weight ratio inhibited A β fibril formation by 62%. A 1:1 A β :catechin hydrate (Aldrich) weight/weight ratio inhibited A β fibril formation by 88%, whereas a 1:0.1 A β :catechin (Aldrich) weight/weight ratio inhibited A β fibril formation by 62%. A 1:1 A β :catechin (Aldrich) weight/weight ratio inhibited A β fibril formation by 85%, whereas a 1:0.1 A β :catechin (Aldrich) weight/weight ratio inhibited A β fibril formation by 66%. This initial data indicated that catechins, including epicatechin, catechin hydrate and catechin were potent inhibitors of Alzheimer's amyloid fibril formation.

Example 5

Inhibition of A β Amyloid Fibril Formation by Catechins as Demonstrated by a Reduction in Congo red Birefringence

The inhibition of A β 1-40 fibril formation by catechins was confirmed by a loss of Congo red birefringence as demonstrated in Congo red staining assays (Figure 6). In these studies, aliquots of 125 μ M A β only, or A β + catechins were incubated for 1 week at 37°C, and then placed on gelatin-coated slides, air-dried overnight and stained with Congo red. A marked reduction in Congo red birefringence (as viewed under polarized light) was exhibited in the presence of epicatechin (ICN)(as an example)(Figure 6) at an A β :epicatechin weight ratio of 1:0.5 (Fig. 6B) and to a lesser extent at an A β :epicatechin weight ratio of 1:0.05 (Fig. 6C). Figure 6A demonstrates the Congo red staining of A β amyloid deposits following incubation of 125 μ M of

A β 1-40 at 37°C for 1 week alone. This study demonstrated that catechins, such as epicatechin, was a potent inhibitor of A β amyloid fibril formation.

Example 6

Disassembly/Disruption of Alzheimer's Disease A β 1-42 Amyloid Fibrils by Catechins

In the next study, commercially available catechins were tested for their ability to cause a disassembly/disruption of pre-formed Alzheimer's disease amyloid fibrils containing A β 1-42. This type of activity would be important for any potential anti-amyloid compound that can be used in patients who already have substantial amyloid deposition in organs and/or tissues. For example, Alzheimer's disease patients in mid-to-late stage disease have abundant A β -containing amyloid deposits in their brains as part of both neuritic plaques and cerebrovascular amyloid deposits. A compound capable of causing disassembly/disruption of pre-existing amyloid deposits would be advantageous for use in these patients who are at latter stages of the disease process.

For this study, 1 mg of A β 1-42 (Bachem Inc., Torrance, CA, USA) was dissolved in 1.0 ml of double distilled water (1 mg/ml solution). 25 μ M of A β 1-42 was then incubated at 37°C, in the absence or presence of epicatechin (from Fluka), epicatechin (from ICN), catechin hydrate (from Aldrich), Gallocatechin gallate (from Sigma), Epicatechin gallate (from Sigma), Epicatechin (from Sigma), Epicatechin green tea (from Sigma) in the presence of 150 mM Tris HCl, 10 mM NaCl (pH 7.0) with 0.02% sodium azide. In these studies (see results described below and in Figure 7), the A β 1-42:catechin weight ratios tested 1:1, 1:0.1 and 1:0.01. The Thioflavin T fluorometry assay as described in Example 1 was used.

As shown in Figure 7, increasing amounts of various commercially available catechins caused a dose-dependent disassembly/disruption of pre-formed Alzheimer's A β 1-42 fibrils. A β 1-42 alone demonstrated a mean fluorescence of 1037 +/- 101 fluorescence units (Figure 7). Epicatechin (from Fluka, ICN, Aldrich and Sigma), catechin hydrate, gallocatechin gallate, and epicatechin gallate all caused a disruption/disassembly of pre-formed A β (1-42) amyloid fibrils in a dose-dependent manner, and significantly ($p < 0.01$) at A β :catechin weight ratios of 1:1 and 1:0.1 (Figure 6). A 1:1 A β :epicatechin (Fluka) weight/weight ratio disrupted A β 1-42 fibril formation by 86%, whereas a 1:0.1 A β :epicatechin (Fluka) weight/weight disrupted A β 1-42 fibril formation by 63%. A 1:1 A β :epicatechin (ICN) weight/weight ratio disrupted A β 1-42 fibril formation by 91%, whereas a 1:0.1 A β :epicatechin (ICN) weight/weight

disrupted A β 1-42 fibril formation by 78%. A 1:1 A β :epicatechin (Aldrich) weight/weight ratio disrupted A β 1-42 fibril formation by 85%, whereas a 1:0.1 A β :epicatechin (Aldrich) weight/weight ratio disrupted A β 1-42 fibril formation by 74%. A 1:1 A β :catechin hydrate (Aldrich) weight/weight ratio disrupted A β 1-42 fibril formation by 86%, whereas a 1:0.1 A β : catechin hydrate (Aldrich) weight/weight ratio disrupted A β 1-42 fibril formation by 60%. A 1:1 A β :galocatechin gallate (Sigma) weight/weight ratio disrupted A β 1-42 fibril formation by 86%, whereas a 1:0.1 A β : galocatechin gallate (Sigma) weight/weight ratio disrupted A β 1-42 fibril formation by 44%. A 1:1 A β :epicatechin gallate (Sigma) weight/weight ratio disrupted A β 1-42 fibril formation by 92%, whereas a 1:0.1 A β : epicatechin gallate (Sigma) weight/weight ratio disrupted A β 1-42 fibril formation by 65%. A 1:1 A β :epicatechin (Sigma) weight/weight ratio disrupted A β 1-42 fibril formation by 85%, whereas a 1:0.1 A β : epicatechin (Sigma) weight/weight ratio disrupted A β 1-42 fibril formation by 81%. A 1:1 A β :epicatechin green tea (Sigma) weight/weight ratio disrupted A β 1-42 fibril formation by 86%, whereas a 1:0.1 A β : epicatechin green tea (Sigma) weight/weight ratio disrupted A β 1-42 fibril formation by 63%. This study demonstrated that the catechins, including epicatechin (from Fluka, ICN, Aldrich and Sigma), catechin hydrate (Aldrich), galocatechin gallate (Sigma), and epicatechin gallate all caused a disassembly/disruption of pre-formed A β 1-42 amyloid fibrils and was effective in a dose-dependent manner.

Example 7

Catechins are Potent Inhibitors of Parkinson's Fibril Formation

In the next study, commercially available catechins were tested for their ability to inhibit Parkinson's alpha-synuclein fibril formation. NAC (also known as, and sometimes referred to herein as, NAC-P), a 35 amino acid fragment of alpha-synuclein was used since it is known to spontaneously form amyloid-like fibrils following incubation at 37°C. For this study, 62.5 μ M of NAC (Bachem Inc., Torrance, CA, USA) was incubated in microcentrifuge tubes at 37°C for 1 week (in triplicate), either alone, or in the presence of catechins from different commercial sources including Epicatechin (EC)(Fluka), Epicatechin (EC)(ICN), Epicatechin (EC)(Aldrich), Epicatechin (EC)(Sigma), Epicatechin gallate (Sigma), Catechin hydrate (Aldrich), and Galocatechin gallate (Sigma) in 150 mM Tris HCl, 10 mM NaCl, pH 7.0 (TBS). Increasing amounts of catechins (as described above) were tested including NAC:catechin at a 1:1, 1:0.5, 1:0.1, and 1:0.01 weight/weight ratio to determine if

catechins exerted a dose-dependent inhibition of NAC fibril formation. To assess the effects of catechins on NAC fibril formation, aliquots were taken from each tube for analysis at 0, 3 days, and 7 days using the Thioflavin T fluorometry procedure as described in Example 4.

5 The effects of commercially available catechins on NAC fibril formation was evaluated over a 1-week incubation period. During the 1-week incubation period, there was a gradual increase in the fluorescence of NAC, with a peak fluorescence of 381 +/- 50 fluorescence units observed at 7 days (Figure 8). Epicatechin (from Fluka, ICN, Aldrich and Sigma), epicatechin gallate (Sigma), catechin hydrate (Aldrich), and
10 gallo catechin gallate (Sigma) all inhibited NAC fibril formation in a dose-dependent manner, and significantly ($p < 0.01$) at NAC:catechin weight ratios of 1:1, 1:0.5, 1:0.1 and 1:0.01 (Figure 8). A 1:0.1 NAC:epicatechin (Fluka) weight/weight ratio inhibited NAC fibril formation by 70%, whereas a 1:0.01 NAC:epicatechin (Fluka) weight/weight ratio inhibited NAC fibril formation by 46%. A 1:0.1 NAC:epicatechin (ICN)
15 weight/weight ratio inhibited NAC fibril formation by 89%, whereas a 1:0.01 NAC:epicatechin (ICN) weight/weight ratio inhibited NAC fibril formation by 49%. A 1:0.1 NAC:epicatechin (Aldrich) weight/weight ratio inhibited NAC fibril formation by 76%, whereas a 1:0.01 NAC:epicatechin (Aldrich) weight/weight ratio inhibited NAC fibril formation by 33%. A 1:0.1 NAC:epicatechin (Sigma) weight/weight ratio inhibited
20 NAC fibril formation by 75%, whereas a 1:0.01 NAC:epicatechin (Sigma) weight/weight ratio inhibited NAC fibril formation by 40%. A 1:0.1 NAC:epicatechin gallate (Sigma) weight/weight ratio inhibited NAC fibril formation by 77%, whereas a 1:0.01 NAC:epicatechin gallate (Sigma) weight/weight ratio inhibited NAC fibril formation by 34%. A 1:0.1 NAC:catechin hydrate (Aldrich) weight/weight ratio inhibited NAC
25 fibril formation by 77%, whereas a 1:0.01 NAC:catechin hydrate (Aldrich) weight/weight ratio inhibited NAC fibril formation by 34%. A 1:0.1 NAC:gallo catechin gallate (Sigma) weight/weight ratio inhibited NAC fibril formation by 73%, whereas a 1:0.01 NAC:gallo catechin gallate (Sigma) weight/weight ratio inhibited NAC fibril formation by 34%. This study demonstrated that the catechins, including epicatechin
30 (from Fluka, ICN, Aldrich and Sigma), catechin hydrate (Aldrich), gallo catechin gallate (Sigma), and epicatechin gallate (Sigma) all caused an inhibition of NAC fibril formation and was effective in a dose-dependent manner.

Example 8

Screening and Identifying Specific Polyhydroxylated Aromatic Compounds as Inhibitors of AA Amyloidosis Using a Cell Culture Model System

5 It is believed that specific polyhydroxylated aromatic compounds are potent inhibitors/disrupters of fibrillar AA amyloid formation and deposition in cell culture. A group of about 30 commercially available polyhydroxylated aromatic compounds that differ by position of various functional hydroxyl groups are believed to inhibit fibrillar AA amyloid formation and deposition using a cell culture model system. A murine
10 macrophage cell culture system receiving recombinant serum amyloid A 2 (SAA2) and amyloid enhancing factor (AEF) is used to generate fibrillar AA amyloid *in vitro*. Fibrillar AA amyloid deposition detected by Congo red staining and Thioflavin S fluorescence is quantitated using an image analysis system. In addition, quantitation
15 of AA amyloid formation and deposition is achieved by the analysis of the cell layer and media derived from cultures using SDS-PAGE, immunoblotting and scanning densitometry. Selected efficacious compounds found to inhibit AA amyloid formation and/or deposition in culture are then tested in an experimental mouse model of AA amyloidosis.

Example 9

Determining the Efficacy of Selected Polyhydroxylated Aromatic Compounds in a Mouse Model of Systemic AA Amyloidosis

 It is believed that specific polyhydroxylated aromatic compounds are potent inhibitors of fibrillar AA amyloid formation and deposition in a mouse model of
25 experimental AA amyloidosis. Selected efficacious compounds identified as above are used to orally treat (by gavage) groups of CBA/J mice (n=8 per group) over a 21-day treatment period. Fibrillar AA amyloid is induced in these animals on day 8 of the treatment period by a single subcutaneous injection of silver nitrate (inflammatory stimulus), followed by a single tail-vein injection of 100µg AEF. A group of 12 animals
30 orally treated with phosphate-buffered saline (PBS) and induced for AA amyloidosis (on day 8) serve as the control group. Spleen, liver and kidneys from each animal are obtained and the % amyloid burden in each organ are quantified using image analysis following Congo red staining or Thioflavin S fluorescence. In addition, immunohistochemistry is implemented for the detection of AA amyloid protein and its
35 precursor (i.e. SAA), as well as other known AA amyloid-associated components (i.e. heparan sulfate proteoglycans/glycosaminoglycans, amyloid P component, and ApoE)

to determine if reduction of AA amyloid deposition in tissues also correlates with reduction in amyloid-associated components.

We have established the use of the experimental AA mouse model in-house. AEF was first kindly provided by Dr. Robert Kisilevsky (Queen's University, Kingston, Ontario, Canada). CBA/J female mice were then injected subcutaneously with a single 0.5ml injection of 3% silver nitrate (to induce an inflammatory reaction) and a single tail-vein injection of 100µg AEF. Using this model within 2-3 days following injection, abundant AA amyloid deposition occurred in spleen, followed by liver and kidney. As shown in Figure 9, fibrillar AA amyloid deposits were first observed in the perifollicular areas of the spleen. Congo red staining of tissue sections revealed a characteristic red/green birefringence (as viewed under polarized light) indicative of fibrillar amyloid deposits (Fig. 9A). The presence of fibrillar amyloid deposits was also confirmed by positive Thioflavin S fluorescence (Fig.9C).

Confirmation of AA amyloid deposits in spleen, liver and kidney was demonstrated by positive immunostaining using a polyclonal antibody against the AA amyloid protein (Serotek)(not shown). In addition, as previously described in a number of studies (Snow *et al*, Lab. Invest. 53:37-44, 1985; Snow *et al*, Lab. Invest. 56:665-675, 1987; Snow *et al*, Lab. Invest. 57:687-698, 1987; Snow *et al*, J. Histochem. Cytochem. 39:1321-1330, 1991.) AA amyloid deposits demonstrated a co-localization of heparan sulfate glycosaminoglycans (likely as part of proteoglycans) as shown using a phage display generated periplasmic fraction antibody (known as HS4C3 which recognizes a specific heparan sulfate GAG epitope)(van Kuppevelt *et al*, J. Biol. Chem. 273:12960-12966,1998.)(Fig. 9C).

Figure 9 shows amyloid deposition in spleen in a mouse model of experimental AA amyloidosis. Panels represent splenic tissue taken 5 days after CBA/J mice were injected with 100µg AEF + 0.5ml of 3% silver nitrate. (Fig. 9A) Splenic amyloid in the perfollicular area (bright areas; arrows) as shown by Congo red staining (i.e. red/green birefringence) as viewed under polarized light. (Fig. 9B) Splenic amyloid (bright areas; arrows) in the perfollicular area in another animal as demonstrated by positive Thioflavin S fluorescence. (Fig. 9C) Co-localization of heparan sulfate glycosaminoglycans in the splenic perfollicular areas (dark areas; arrows) as demonstrated by immunostaining with a phage-display generated antibody (known as HS4C3), that recognizes a specific heparan sulfate GAG epitope. All figures X100.

In the liver, fibrillar AA amyloid deposits were first observed within 3-4 days in the walls of the central veins (Fig. 10A), followed by amyloid deposition throughout the parenchyma by days 5-7 (Fig. 10C). The nature of fibrillar amyloid deposits was confirmed by both positive staining with Congo red (i.e. red/green birefringence as viewed under polarized light)(Figs. 10A, 10C) and Thioflavin S (i.e. positive fluorescence)(Fig. 10B).

Figure 10 shows amyloid deposition in liver in a mouse model of experimental AA amyloidosis. Panels A and B represent liver tissue taken 7-10 days after CBA/J mice were injected with 100µg AEF + 0.5ml of 3% silver nitrate. (Fig. 10A) Liver amyloid (at 7 days) primarily confined to the walls of a central vein (bright areas; arrows) as shown by Congo red staining (i.e. red/green birefringence) as viewed under polarized light (arrows). X100 (Fig. 10B) Liver amyloid (at 7 days) in the walls of central veins (bright areas; arrows) and penetrating into the parenchyma (arrowheads) as detected by Thioflavin S fluorescence. X100 (Fig. 10C) A lower magnification photomicrograph demonstrating marked amyloid accumulation (at 10 days) in the walls of central veins (bright areas; arrowheads) and throughout the parenchyma in liver following 10 days after injection of AEF + AgNO₃. X25.

These studies indicate that the experimental mouse model of systemic AA amyloidosis is viable. We utilize this model to test the efficacy of AA amyloid inhibitors following initial screening using the cell culture model system.

Example 10

Identification of a New Class of Compounds as Potential Anti-Amyloid Agents

Most of our previous studies involved the analysis of these compounds for the disruption/disassembly of pre-formed amyloid fibrils consisting of beta-amyloid protein (observed in Alzheimer's disease amyloid deposits), islet amyloid polypeptide (observed in the islets of 90% of patients with type 2 diabetes), alpha-synuclein (observed in Parkinson's disease deposits), or prion protein (observed in prion diseases). Our initial studies demonstrate that specific polyphenolic compounds can cause disruption/disassembly of different types of amyloid proteins, and thus tend to be less specific, whereas other polyphenolic compounds tend to only disrupt one type of amyloid protein (and are thus more specific). Examples of some of the polyhydroxylated aromatic compounds which we have screened for inhibition of AA amyloid formation and deposition are set out below.

As an example, Figure 11 demonstrates the effect of specific polyhydroxylated aromatic compounds to disrupt/disassemble amyloid fibrils consisting of islet amyloid polypeptide (IAPP)(the amyloid protein that accumulates in the pancreatic islets of ~90% of patients with type 2 diabetes)(Westermarck, 1972; Clark et al, 1988) as assessed by Thioflavin T fluorometry (Naiki et al, 1991; Levine III, 1993; 1995). In this assay, increases or decreases in Thioflavin T fluorometry is proportional to increases or decreases in the quantity of IAPP fibrils (Naki et al, 1991; Levin III, 1993; 1995). As shown in Figure 11, following a 1-week incubation at 37°C, 25 µM of IAPP alone demonstrated 5,806 +/- 301 fluorescence units indicating the presence of abundant IAPP amyloid fibrils. IAPP fibrils incubated for 1 week at 37°C at an IAPP: compound weight ratio of 1:0.1 (approximate molar ratio of 1:1) with specific hydroxylated aromatic compounds including epicatechin (EC; Fluka), epicatechin gallate (ECG; Sigma) or pyrocatechol (Sigma) all demonstrated a marked 90-96% disruption/disassembly of pre-formed IAPP fibrils. On the other hand, EDTA (as a negative control) and the polyphenolic known as quinic acid did not cause any significant disruption/disassembly of pre-formed IAPP fibrils (Fig. 11). The direct disruption of pre-formed IAPP fibrils by specific polyphenolic compounds was also confirmed by Congo red staining assays (not shown).

Figure 11 shows specific polyhydroxylated aromatic compounds act as potent disruptors of pre-formed amyloid fibrils. This graph is a 1-week Thioflavin T fluorometry assay to identify inhibitors of IAPP fibrils. As shown, only epicatechin (EC), epicatechin gallate (ECG) and pyrocatechol disrupt/disassemble pre-formed IAPP fibrils by 90-96%.

In our other studies, circular dichroism spectroscopy is utilized to test the efficacy of certain polyphenolics to disrupt pre-formed fibrils consisting of beta-amyloid protein (Aβ) 1-42, a protein that forms amyloid fibrils as part of extracellular amyloid plaques in the brains of all patients with Alzheimer's disease. As an example, the effects of the polyphenolic, epicatechin on the disruption of Aβ 1-42 fibrils was tested. In this study, 50µM of Aβ 1-42 (Bachem Inc) was incubated in the absence or presence of epicatechin (EC)(Sigma) for 1 week at 37°C, at an Aβ:EC wt/wt ratio of 1:1 (approximate molar ratio of 1:10). As shown in Figure 12, Aβ 1-42 alone demonstrates the typical spectra of a fibrillar amyloid protein containing β-pleated sheet structure by the marked minima observed at 222 nm. On the other hand, incubation with epicatechin by 3 days caused a significant disruption/disassembly of β-pleated sheet

structure (Fig. 12), suggesting that specific polyhydroxylated aromatic compound's mechanism of action may involve β -pleated sheet disruption.

Figure 12 shows disruption/disassembly of β -pleated sheet structure of pre-formed Alzheimer's disease amyloid fibrils by epicatechin. Circular dichroism spectroscopy was used to assess the ability of epicatechin (EC) to cause a disruption/disassembly of β -pleated sheet structure of pre-formed A β 1-42 fibrils. As shown, by 3 days of co-incubation epicatechin was effective in disrupting the β -sheet structure (as revealed by the change in the spectra, especially at 222 nm).

Example 11

10 **Inhibition of Experimental AA Amyloid Amyloidosis by Epicatechin**

Studies also demonstrate that the specific polyhydroxylated aromatic compound, known as epicatechin (C₁₅H₁₄O₆; MW 290.3) has the ability to markedly prevent AA amyloid deposition in spleen and liver in the mouse model (i.e. following AEF + silver nitrate administration). This was not observed following treatment with catechin (the epimer of epicatechin), suggesting that structure-activity relationships exist for the inhibition of AA amyloid fibril formation and deposition by polyhydroxylated aromatic compounds.

In this study, 4 groups (n= 6 per group) of female CBA/J 8-week old mice were first pre-treated for 7 days with either a) phosphate-buffered saline (PBS), b) epicatechin (C₁₅H₁₄O₆; MW 290.3)(Fluka, St. Louis, MO) or c) catechin hydrate (C₁₅H₁₄O₆; MW 290.3)(Fluka, St. Louis, MO). Epicatechin was administered daily by oral gavage (0.5 ml of test article in PBS, pH 7.4) at a dosage of 78 mg/kg/day (solubility limit of epicatechin), whereas catechin hydrate was administered at a high dose (500 mg/kg/day).

Following a 7-day pretreatment period with either PBS, epicatechin or catechin, all animals were induced for AA amyloid deposition by a single subcutaneous injection of 0.5 ml of silver nitrate (inflammatory stimulus), followed by a single tail vein injection of 100 μ g of AEF in distilled water. The mice were then treated for another 14 days with PBS, epicatechin or catechin by oral gavage as described above (total test compound treatment time of 21 days). At the end of the experimental protocol, the animals were sacrificed and the spleen, liver and kidneys were taken for histological analysis.

Tissue sections (i.e. spleen, liver and kidney) were then stained for fibrillar amyloid using both Congo red and Thioflavin S. Five sections per organ was then used

to evaluate amyloid load. In these studies, an arbitrary scoring system was used (from 0 to 5) and amyloid load per organ was determined by the blind scoring of two investigators. As an example, for Congo red scoring of tissue sections in spleen, 0 = no Congo red birefringence in tissue; 1= slight Congo red birefringence confined to one portion of the perifollicular area; 2= Congo red birefringence in confined to two or more portions of the prefollicular area; 3 = Congo red birefringence occupying the entire perifollicular area; 4= Congo red birefringence occupying the entire perifollicular area and penetrating into the white pulp; 5=Congo red birefringence occupying the entire perifollicular area and penetrating deep into white pulp. We also use a more quantitative approach to determine % amyloid load in various tissues using a more sophisticated image analysis system.

As shown in Figure 13, animals given saline alone demonstrated marked amyloid deposition in spleen by the end of the 2-week amyloid induction period, with a Congo red score of 3.83 +/- 0.1. Catechin hydrate (i.e. Congo red score of 3.20 +/- 0.46) reduced AA amyloid deposition in spleen by 16.5%, but this was not significant. However, epicatechin (i.e. Congo red score of 2.16 +/- 0.50) significantly ($p < 0.01$) inhibited fibrillar AA amyloid deposition in spleen by 43.7% (Fig. 13). A similar inhibition of fibrillar AA amyloid in liver and kidney was also found following treatment with epicatechin, but not catechin (not shown).

The inhibitory effect by epicatechin is so potent, that a few of the animals even demonstrated little to no fibrillar AA amyloid in tissues following treatment (Figure 14). This study demonstrates that selected polyhydroxylated aromatic compounds have the ability to serve as potential therapeutics for the treatment of systemic AA amyloidosis. The studies described use cell culture techniques and an experimental mouse model of AA amyloidosis to identify polyhydroxylated aromatic compounds that serve as lead compounds for the treatment of AA amyloidosis.

Figure 14 shows a marked reduction in splenic AA amyloid deposition by epicatechin as revealed by Congo red staining. (Fig. 14A) Congo red staining of AA amyloid deposits in the perifollicular area of the spleen (arrows) in a mouse induced with AEF + AgNO₃ (on day 8), and treated with PBS (for 21 days). Color photomicrographs demonstrate a red/green birefringence indicative of fibrillar amyloid (as viewed under polarized light). (Fig. 14B) Near complete elimination of AA amyloid deposits in the perifollicular area of the spleen (arrows) as shown by lack of Congo red

staining (as viewed under polarized light) of an animal induced with AEF + AgNO₃ (day 8), and treated with epicatechin (for 21 days).

Example 12

Identification of New AA Amyloid Inhibitors – In Vitro Screening

5 Our studies suggests that certain polyhydroxylated aromatic compounds, such as epicatechin, may serve as a potent inhibitor of fibrillar AA amyloid formation and deposition in tissues. We use innovative cell culture techniques to screen a number of known and commercially available polyhydroxylated aromatic compounds which differ by the number of aromatic rings and/or by the position of various hydroxyl and other
10 function groups on the aromatic rings. These structure-activity relationship (SAR) type of screening studies give insight into the important structural characteristics needed for effective inhibition of AA amyloid formation and deposition. A murine macrophage culture system utilizing recombinant SAA2 and AEF is used to generate fibrillar AA amyloid *in vitro*. Fibrillar AA amyloid deposition detected by Congo red staining and
15 Thioflavin S fluorescence is quantitated in culture using an image analysis system equipped for fluorescence and polarization. In addition, quantitation of AA amyloid formation is achieved by the analysis of the cell layer and media using SDS-PAGE, immunoblotting and scanning densitometry. When AA amyloid is established in culture, cultures are screened with selected polyhydroxylated compounds at increasing
20 concentrations. The most efficacious compounds found to inhibit AA amyloid formation and deposition in culture are then tested in a relevant experimental mouse model of AA amyloidosis.

Methodology:

Establishment of a Cell Culture System for the Study of AA Amyloidosis

25 For the establishment of a cell culture system in which to screen for inhibitors of fibrillar AA amyloid formation and deposition, we use the method as described by Kluve-Beckerman et al (1999).

Collection and Culture of Murine Macrophage/Peritoneal Cells:

30 Cells are collected by lavage from the peritoneal cavity of normal 8-10 week old female C57Bl/6 mice. Briefly, 8 ml of collection medium (RPMI 1640; Life Technologies, Grand Island, NY), 25 mM HEPES, pH 7.0 and 1X antibiotic-antimycotic (Life Technologies) is injected intraperitoneally, the abdomen is gently massaged, and the fluid and cells are withdrawn. Cells are collected by centrifugation, resuspended at a concentration of 5X10⁶ cells/ml, and plated at a density of 1.7 X 10⁶ cells/well in 8-well

chamber slides. Culture media contain RPMI 1640, 2 mM L-glutamine, 1X antibiotic-antimycotic, and 15% fetal calf serum (Hyclone Labs, Logan, VT). Cells are allowed to attached for 3 hours, after which wells are rinsed thoroughly to remove nonadherent cells. Adherent cells display a uniform, round morphology typical of freshly plated, nonactivated macrophages. Cells are maintained in 350µl of culture medium per well at 37°C in an atmosphere of 5% CO₂, with media being replaced every 2-3 days. Following the addition of AEF and recombinant SAA2 (as described below) for the induction of AA amyloid deposition in culture (usually observed within 2-4 days of induction), cell cultures are maintained for a period of 14-20 days.

Preparation of Amyloid Enhancing Factor:

AEF is prepared from the spleens of amyloidotic mice following a 2-week treatment with a single subcutaneous 0.5ml injection of AgNO₃, and a single tail vein injection of 100µg AEF in distilled water. The initial AEF material was kindly supplied by Dr. Robert Kisilevsky (Queen's University, Canada) to which more AEF was generated using the method of Pras et al (1968). Briefly, approximately 2 grams (10 amyloidotic spleens) of tissue is processed for each preparation. The spleens are homogenized using a Kontes tissue grinder in 40mls of 0.15M NaCl saline solution, until the tissue is in a fine slurry. The slurry is then centrifuged on a table-top microcentrifuge at 10,000 rpm for 30 minutes. The supernatant is discarded and the pellet is resuspended in 40mls of saline and centrifuged again as described above. This process is repeated seven times. The salt is then be removed from the pellets by rehomogenizing in sterile distilled water, and centrifugation on a table-top microcentrifuge at 15,000 rpm for 2 hours. The supernatant is then discarded and the pellet is resuspended in 20mls of distilled water, and then recentrifuged at 15,000 rpm for 2 hours. This time the supernatant is not discarded and is designated as Supernatant II (Sup II), which contains significant AEF activity. The process used for the generation of Sup II is repeated, and the supernatant, designated as Sup III is saved. The pellet obtained from the last step is resuspended in 7 mls of distilled water and centrifuged at 10,000 rpm for 3 hours. The supernatant is saved and designated as Sup IV. Sup II, Sup III, and Sup IV contain AEF activity which is assayed by the Bradford method (Bradford, 1976) to determine the protein concentration for each supernatant. The 3 supernatants are then pooled and lyophilized such that multiple 100µg lyophilized aliquots are obtained. AEF is used for both cell culture studies (aim 1) and experimental AA amyloid induction in a mouse model (aim 2) as described

below. We have found that 10 amyloidotic spleens yield approximately 4 mg of AEF (enough for injections for 40 animals; i.e. 100µg per animal as described herein.

Production and Purification of Recombinant SAA2:

Mouse recombinant SAA2 corresponding to the SAA in mice of the CE/J strain (CE/J SAA) is produced in Escherichia coli BL834 cells, using the pET-21a vector as previously described (Kluve-Beckerman et al, 1997). Purification from E. coli lysates are accompanied by Sepharose CL-6B chromatography in 4 M guanidine, 0.05 M Tris-HCl (pH 8.2) followed by chromatofocusing over a range of pH 8 to pH 5 in 6M urea (Kluve-Beckerman et al, 1997). Final preparations are precipitated in ammonium sulfate (80% saturated), extensively dialyzed against water, and lyophilized.

Induction of AA Amyloid Formation in Cell Culture:

To induce AA amyloid formation and deposition in macrophage/peritoneal cells in culture, recombinant mouse SAA2 (at a final concentration of 140µg/ml), and AEF (at a final concentration of 12 µg/ml) is used. SAA (7µl) is added directly to the medium (350µl) from a 7-10 mg/ml stock solution prepared by dissolving purified, lyophilized recombinant SAA2 in 6M urea, 25 mM HEPES, pH 7.2. Concentrations of stock solutions are adjusted after analysis by SDS-PAGE and densitometric quantitation of Coomassie-blue stained SAA bands. AEF stock solutions (2 mg/ml) is thoroughly mixed to resuspend precipitated material before withdrawal of an aliquot (2µl) for addition to culture medium (350µl).

Cell Viability Assay:

To confirm cell viability of macrophage cultures following the induction of AA amyloid (as described above), exclusion of trypan blue is used in some cultures (at days 5, 10 and 20). Before staining, cells are rinsed 3 times with serum-free RPMI. They are then covered for 1 minute with a solution of 2% (w/v) trypan blue in PBS (Perry et al, 1997). Immediately after the trypan blue is removed, cells are fixed with 4% (w/v) paraformaldehyde, pH 7.5, for 10 minutes at room temperature, rinsed four times with PBS with gentle shaking, and examined microscopically before staining with Congo red or Thioflavin S.

Macrophage Phagocytotic Activity:

In order to assess and confirm phagocytotic activity of macrophage cells in culture (at days 5, 10 and 20 following AA amyloid induction), a latex bead uptake assay is also employed. Blue-dyed polystyrene latex beads, 0.8µm in diameter (Sigma) is suspended in serum-free RPMI at a concentration of 7.2×10^8 /ml. Cells are rinsed

3 times with serum-free RPMI and then incubated for 30 minutes with 300µl of the bead suspension. During the incubation, chamber slides are tightly wrapped in Parafilm and shaken gently in a water bath at 37°C. The bead suspension is removed, and the cells are rinsed a minimum of 10 times with RPMI. Cells are then fixed with formalin and stained with hematoxylin.

Congo Red Staining:

To confirm and quantitate fibrillar AA amyloid deposition in culture, cells at 5, 10 and 20 days (post AA amyloid induction) are fixed in ice-cold 100% methanol and then stained for 45 minutes with Congo red prepared in alkaline 80% ethanol (Puchtler et al, 1962). After several quick dips in water, slides are immersed in hematoxylin for 2 minutes. Slides are then dipped once in acidified 70% ethanol, several times in water, and once in a 1% solution of NaOH. Dehydration is accomplished by washing sequentially in 95% ethanol and 100% ethanol. Slides are cleared in xylene, and coverslips are applied using Permount. The extent of Congo red staining (i.e. red/green birefringence as viewed under polarized light) is quantitatively determined using an imaging system as described herein. Previous studies using this culture system indicated that Congo red-positive material remains attached to chamber slides throughout the culture period (2-24 days) (Kluve-Beckerman et al, 1999). AA amyloid accumulation in culture has been previously demonstrated to occur within 2 days following addition of SAA2 and AEF (Kluve-Beckerman et al, 1999).

Thioflavin S Fluorescence:

Fibrillar AA amyloid deposits are detected and analyzed following staining with Thioflavin S (Elghetany and Saleem, 1988). Thioflavin S fluorescence is quantitated using the image analysis system as described herein.

Quantitation of %AA Amyloid Burden in Cell Culture by Image Analysis:

In order to quantitate the % amyloid burden present in cell cultures following AA amyloid formation and deposition (at days 5, 10, and 20), we use a quantitative method involving image analysis. Briefly, slides stained with Congo red or Thioflavin S are viewed under polarized light, or fluorescent light, respectively. 5-7 slides per treatment group as described herein are used to determine the % amyloid burden at a given magnification (X100). Quantitative image analysis is performed using an Image Pro Plus image analysis system (Media Cybernetics) linked to a Zeiss Axioskop 20 microscope (with polarization and fluorescence capabilities) through a CCD video camera. The images of fibrillar AA amyloid (polarized or fluorescent) sections are

stored in a buffer and a specific region (i.e. at a magnification of X100) is manually outlined and the total pixel area occupied by the amyloid structures determined. A monochromatic-based threshold is used to select pixels corresponding to polarized or immunofluorescent structures. The % of the region occupied by the labeled pixels (i.e. red/green for Congo red; yellow/green for Thioflavin S) are calculated. For all image analyses, the treatment status of cultures are unknown to the observer. Mann-Whitney nonparametric analysis is performed using Statview software (SAS Institute, Cary, NC). Reductions of amyloid loads caused by treatment with polyhydroxylated aromatic compounds (as described below) can be quantitatively determined using the above described methodology. We have successfully utilized this imaging system (using Congo red staining; Thioflavin S fluorescence; or a biotinylated-A β antibody) to determine % amyloid load, number of amyloid plaques, and plaque size, in various brain regions in transgenic mouse models of Alzheimer's disease. Using this method, we also quantitate the number of macrophages *in vitro* (at a magnification of X100) that contain amyloid-laden structures. Previous studies by Kluve-Beckerman et al (1999) demonstrate that using the cell culture system as described above, many of the macrophages accumulate fibrillar AA amyloid within the cytoplasm of macrophage cells.

Testing of Polyhydroxylated Aromatic Compounds for Inhibition of AA Amyloid Formation and Deposition:

Our data suggests that certain polyhydroxylated aromatic compounds possess potent amyloid inhibitory activity. Commercially available polyphenolic compounds believed suitable for inhibition of fibrillar AA amyloid formation and deposition are disclosed below.

For structure-activity relationship studies, these readily available polyphenolic compounds with hydroxy groups situated at various positions on aromatic rings may be purchased from commercial sources. Initial studies testing the toxicity of these compounds indicate that they usually demonstrate limited toxicity, even at high dosages. Quantitation of % AA amyloid burden as described herein is used to assess the ability of various polyhydroxylated aromatic compounds to inhibit/disrupt AA amyloid formation and deposition in culture.

Representative compounds disclosed and believed to be effective anti-amyloid and anti alpha-synuclein / NAC inhibitors / disrupters include EDTA (C₁₀H₁₆N₂O₈ MW 292.2 used as a negative control only), and commercially available polyphenolic

compounds with a variety of phenolic substitution patterns including: Epicatechin ($C_{15}H_{14}O_6$; MW 290.3), Catechin hydrate ($C_{15}H_{14}O_6$; MW 290.3), Epigallocatechin ($C_{15}H_{14}O_7$; MW 306.3), Epigallocatechin Gallate ($C_{22}H_{18}O_{11}$; MW 458.4), Epicatechin Gallate ($C_{22}H_{18}O_{10}$; MW 442.4), Gallocatechin Gallate ($C_{22}H_{18}O_{11}$; MW 458.4),
5 Chlorogenic acid ($C_{16}H_{18}O_9$; MW 354.3), Hematoxylin ($C_{18}H_{14}O_6$; MW 302.3), Phloroglucide ($C_{12}H_{10}O_5$; MW 234.2), Propyl gallate ($C_{10}H_{12}O_5$; MW 212.2), Gallic acid ethyl ester ($C_9H_{10}O_5$; MW 198.2), Gallic acid ($C_7H_6O_5$; MW 170.1), 3,4,5,-trihydroxybenzamide hydrate ($C_7H_7NO_3$; MW 169.1), 5-hydroxydopamine ($C_8H_{11}NO_3$; MW 205.6), 1,2,4-benzenetriol ($C_6H_6O_3$; MW 126.1), Ellagic acid ($C_{14}H_6O_8$; MW 302.2), Quercetin
10 ($C_{10}H_{12}O_5$; MW 338.3), Pyrocatechol ($C_6H_6O_2$; MW 110.1), Tannic acid ($C_{76}H_{52}O_{46}$; MW 1701.2), Pyrogallol ($C_6H_6O_3$; MW 126.1), and p-aminosalicylic acid ($C_7H_7NO_3$; MW 153.1).

All of these compounds are commercially available from Sigma (St. Louis, MO), Fluka (St. Louis, MO) or from Acros Organic (subsidiary of Fisher Scientific, Palatine,
15 IL). As an example of SAR type studies we compare compounds that contain three adjacent hydroxyl groups on an aromatic ring (examples include pyrogallol, 5-hydroxydopamine, gallic acid and propyl gallate) to compounds that contain two adjacent hydroxyl groups (examples include pyrocatechol, quercetin and 1,2,4-benzentriol). Compounds that contain hydroxyl groups that are spaced at least 2
20 carbons apart (such as phloroglucide) are also evaluated in cell culture. These studies help determine phenolic structural features that contribute to the inhibition of AA fibrillogenesis formation and/or deposition. Synthesis of chemical analogs to the most potent inhibitors identified herein are also contemplated. These studies provide good insights into the structural elements required for the observed bioactivity.

25 For cell culture studies, an initial high dosage of test compounds (to be determined empirically) is used to determine if the compounds can inhibit AA amyloid formation and/or deposition, as determined by methods as described below and elsewhere herein. For these studies, each polyhydroxylated aromatic compound is added to cultures at the start of AA amyloid induction (i.e. AEF + SAA2
30 administration). Cell culture media containing each individual test compound is replaced every 2-3 days. % amyloid burden are quantitated at days 5 and 10 following AA amyloid induction and test compound treatment. Comparisons are directly made to the % amyloid burden in cultures only induced for AA amyloid deposition (i.e. no test compound treatment). Those identified to show promise as inhibitors of fibrillar

AA amyloid formation and/or deposition at a single high dose, are tested more extensively, and at increasing concentrations (i.e. low dose, medium dose and high dose; to be determined empirically) to confirm a dose-dependent inhibition of fibrillar AA amyloid formation and/or deposition. Selected compounds identified by screening
5 using the cell culture methods as described above, are then tested in an experimental mouse model of AA amyloidosis.

Figure 15 shows structure-activity relationship (SAR) studies of different commercially available polyhydroxylated aromatic compounds. Shown are 8 examples of the structures of different commercially available polyphenolic compounds which
10 differ by the placement of the hydroxyl groups on the aromatic ring to be used for SAR screening studies.

Quantitation of AA Amyloid Using SDS-PAGE and Immunoblotting:

In addition to the quantitation of % amyloid burden in culture following Congo red staining and Thioflavin S fluorescence using image analysis, AA amyloid
15 accumulation in cell layer and media is determined using SDS-PAGE followed by immunoblotting as previously described (Kluve-Beckerman et al, 1999). Briefly, cell layers are rinsed three times with serum-free RPMI, scraped into PBS, pelleted by centrifugation, and solubilized in SDS sample buffer. For analysis of AA amyloid in culture media derived from cells, 15µl aliquots of media is added directly to SDS
20 sample buffer. Triplicate samples are subjected to tricine-SDS-PAGE as previously described (Schagger and von Jago, 1987). Using this method, separating spacing, and stacking layers contain 16.5%, 10% and 4% polyacrylamide, respectively. A monoclonal antibody against AA amyloid protein (Serotek), and the technique of enhanced chemoluminescence (ECL) Western blotting is employed according to the
25 manufacturer's recommendations (Amersham Life Science). Using the methods as described above, SAA2 usually appears as multiple bands at ~12-14 kDa and higher, whereas AA protein appears as a major band at ~8.5 kDa (Kluve-Beckerman et al, 1999). Initially once the cell cultures are established, N-terminal sequencing of the major band at ~8.5 kDa is implemented to confirm the presence of AA protein. We
30 have previously used the method of electrophoresis, electroelution and amino acid sequencing to identify other peptide sequences for other in-house projects.

Once this system is established, we use scanning densitometry to determine the extent of AA amyloid protein in cell layer and media following induction by SAA2 + AEF (at 5 and 10 days post AA amyloid induction), in the absence or presence of a high

dose of each polyhydroxylated aromatic compound (as specified above). The average of 3 scans per sample (with samples performed on SDS-PAGE in triplicate) is used to determine the levels of AA amyloid protein in cell layer and media under the various conditions. Statistical analysis comparing scanning densitometric analysis in the absence or presence of polyphenolic compounds is based on paired Student's t-tests with data shown as mean \pm S.E. Significance is reported at the 95% ($p < 0.05$), 99% ($p < 0.01$) and 99.9% ($p < 0.001$) confidence levels. ANOVA is also used as necessary.

Example 13

Identification of New AA Amyloid Inhibitors – Animal Studies

Studies suggest that certain polyhydroxylated aromatic compounds, such as epicatechin, appear to serve as potent inhibitor of fibrillar AA amyloid formation and deposition *in vivo*. We display animal model studies to determine the efficacy of selected polyhydroxylated aromatic compounds in an experimental mouse model of AA amyloidosis. Groups of CBA/J mice ($n = 8$ per group) is first pre-treated with different polyhydroxylated aromatic compounds for a 7-day pre-treatment period at a high non-toxic dose (to be determined empirically). Animals are induced for fibrillar AA amyloidosis by a single subcutaneous injection of AgNO_3 , and a single tail vein injection of $100\mu\text{g}$ of AEF. Treatment during the amyloid induction phase with polyhydroxylated aromatic compounds continues for another 14 days before sacrifice (treatment time = 21 days total). Spleen, livers and kidneys are obtained from each animal, and the % amyloid burden in each organ is quantitated using image analysis. In addition, immunohistochemistry is implemented using antibodies against AA amyloid protein, SAA, heparan sulfate proteoglycans/ glycosaminoglycans, amyloid P component, and ApoE to assess the effects of test compounds on the accumulation AA amyloid/SAA, and other known AA amyloid co-factors.

Methodology:

Treatment Groups:

Groups ($n = 8$ per group) of 8-week old CBA/J female mice (Baxter Labs) is used for the animal model studies. Initially each animal is pre-treated daily by oral gavage (in a 0.5 ml volume) with a specific polyhydroxylated aromatic compound (selected from results of testing above) at an initial high non-toxic dose (to be determined empirically) for a 7-day pretreatment period. Typically our previous low to high dosages with polyhydroxylated aromatic compounds given orally, range from a low dose of 25 mg/kg/day, to a high dose of 500 mg/kg/day. Equivalent dosages are used to

compare different polyhydroxylated aromatic compounds taking into account compound toxicity and compound solubility (in PBS). Oral gavage is accomplished using a 22-gauge oral dosing needle (Popper) of test compounds dissolved in a 0.50 ml of PBS (pH 7.4). On day 8, all animals are induced for AA amyloidosis by a single subcutaneous injection of 0.5ml of 3% AgNO₃ (Fisher) in double-distilled deionized water. This is followed by a single tail-vein injection of 100µg of AEF in double-distilled deionized distilled water. Animals are treated for an additional 14 days with each of the specific polyhydroxylated compounds. One group of 12 mice is induced for AA amyloidosis (by AgNO₃ + AEF as described above) and is treated orally with PBS (pH 7.4) only, in the absence of any test compounds. At the end of the 21-day treatment period, animals are sacrificed by overdose with 0.1ml of euthosol, and the spleen, liver and kidneys from each animal is harvested for staining and immunohistochemistry. The number of animals per group (n=8 for test compounds; n=12 for control saline group) are determined from data obtained, and power calculations based on an alpha level of 0.05 (two-sided) and standard deviations of 0.5, which gave a power of 94%.

AEF Production:

AEF is prepared as described above.

Fixation and Tissue Preparation:

Fixation and tissue preparation is as previously described (Snow et al, 1991). Briefly, the spleen, liver and kidneys are removed from each animal, fixed in 90% ethanol, and 10% formaldehyde for 24 hours at -20°C, embedded in paraffin and sectioned at 25µM (thick sections needed for amyloid load quantitation as described below). Approximately 50-75 sections are cut per animal.

Congo Red Staining and Thioflavin S Fluorescence:

Fibrillar AA amyloid accumulation is detected by Congo red staining (Puchtler et al, 1962) and Thioflavin S fluorescence (Elghetany and Saleem, 1988) as described above.

Quantitation of Amyloid Burden in Tissues:

Image analysis is also used to quantitate the % amyloid burden present in spleen, liver and kidney, of each animal. The % amyloid load in each organ is quantified as assessed following staining with Congo red (as viewed under polarized light) or Thioflavin S (as viewed under fluorescent light). 5-7 equally spaced slides per animal per stain (in each treatment group) are used to determine the % amyloid load

at a given magnification (X100). Quantitative image analysis of amyloid load in a given organ (and at a specific magnification) is performed using the image analysis system as described above. Potential reductions of amyloid load is quantitatively determined for each polyhydroxylated aromatic compound for each organ/animal. Certain test compounds are expected to reduce amyloid load by 40-60%.

Immunohistochemistry:

Tissue sections are also immunostained to detect important AA amyloid protein markers, and known AA amyloid-associated co-factors. A monoclonal antibody against the AA amyloid protein (Serotek) is used to confirm the location of AA amyloid deposits, whereas a polyclonal against SAA (Serotek) is used to detect the precursor protein, SAA. The presence of perlecan (a specific heparan sulfate proteoglycan implicated in AA amyloidosis)(Snow et al, 1991) is detected using a monoclonal antibody against the perlecan core protein (known as HK-102; generous gift of Dr. Koji Kimata, Japan)(Snow et al, 1994), whereas a phage display generated periplasmic fraction antibody (known as HS4C3; generous gift of Dr. Van Kuppevelt)(van Kuppevelt et al, 1998) against a specific epitope of heparan sulfate GAG chains are used to determine the co-localization of heparan sulfate GAGs. Amyloid P component is detected using a polyclonal antibody (Research Diagnostics), whereas ApoE is detected using a polyclonal antibody (Research Diagnostics).

For antibody localization we use standard fluorescence techniques (Basgen et al, 1989; Mosedale et al, 1996). The primary antibody for immunohistochemical staining is used through a series of dilutions to obtain the best specificity with the least background staining. Controls for positive immunostaining consist of sections treated with either 1) preabsorption of the primary antibody with excess antigen (if available), 2) a different primary antibody of the same Ig class and species, and/or 3) PBS + 1% bovine serum albumin instead of the primary antibody (to ensure that there is no non-specific binding of the secondary antibodies employed).

Pharmacology and Utility

The disclosed compounds act to inhibit or prevent amyloid fibril formation, inhibit or prevent amyloid fibril growth, and/or cause disassembly, disruption, and/or disaggregation of preformed amyloid fibrils and amyloid protein deposits. Their activity can be measured *in vitro* by methods such as those discussed in Examples 1 through 4, while their activity *in vivo* against amyloidoses can be measured in animal

models, such as those of Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and others.

The compounds also act to inhibit or prevent alpha-synuclein/NAC fibril formation, inhibit or prevent alpha-synuclein/NAC fibril growth, and/or cause
5 disassembly, disruption, and/or disaggregation of preformed alpha-synuclein/NAC fibrils and alpha-synuclein/NAC-associated protein deposits. Their activity can be measured *in vitro* by methods such as those discussed in Example 4 above.

The therapeutic ratio of a compound can be determined, for example, by comparing the dose that gives effective anti-fibril (anti-amyloid or anti-alpha-synuclein/NAC activity in a suitable *in vivo* model in a suitable animal species such
10 as the mouse, with the dose that gives significant weight loss (or other observable side-effects) in the test animal species.

Pharmaceutical compositions and administration

In general, compounds are administered in pure isolated form in
15 therapeutically effective amounts by any of the usual modes known in the art, either singly or in combination with at least one other compound and/or at least one other conventional therapeutic agent for the disease being treated. A therapeutically effective amount may vary widely depending on the disease, its severity, the age and relative health of the animal being treated, the potency of the compound(s), and other
20 factors. As anti-fibril agents, therapeutically effective amounts of compounds may range from 10-1000mg/Kg body weight; for example, 10-100 mg/Kg body weight. A person of ordinary skill in the art will be able without undue experimentation, having regard to that skill and this disclosure, to determine a therapeutically effective amount of a compound of this invention for the treatment of amyloidosis.

25 In general, compounds are administered as pharmaceutical compositions by one of the following routes: oral, topical, systemic (e.g. transdermal, intranasal, or by suppository), or parenteral (e.g. intramuscular, subcutaneous, or intravenous injection). Compositions may take the form of tablets, pills, capsules, semisolids, powders, sustained release formulations, solutions, suspensions, elixirs, aerosols, or
30 any other appropriate compositions; and comprise at least one compound in combination with at least one pharmaceutically acceptable excipient. Suitable excipients are well known to persons of ordinary skill in the art, and they, and the methods of formulating the compositions, may be found in such standard references as Alfonso AR: Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing

Company, Easton PA, 1985. Suitable liquid carriers, especially for injectable solutions, include water, aqueous saline solution, aqueous dextrose solution, and glycols.

In particular, the compound(s) can be administered, orally, for example, as tablets, troches, lozenges, aqueous or oily suspension, dispersible powders or granules, emulsions, hard or soft capsules, or syrups or elixirs. Compositions intended for oral use may be prepared according to any method known in the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavoring agents, coloring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations.

Tablets contain the compound in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for the manufacture of tablets. These excipients may be for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, maize starch or alginic acid; binding agents, for example, maize starch, gelatin or acacia, and lubricating agents, for example, magnesium stearate or stearic acid or tale. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glycerol monostearate or glycerol distearate may be employed. Formulations for oral use may also be presented as hard gelatin capsules wherein the compound is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the compound in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents may be naturally occurring phosphatides, for example lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example, heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids

such as hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters from fatty acids and a hexitol anhydrides, for example, polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example, ethyl or n-propyl p-hydroxybenzoate, one or
5 more coloring agents, one or more flavoring agents, or one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions may be formulated by suspending the compound in a vegetable oil, for example arachis oil, olive oil, sesame oil, or coconut oil or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent, for
10 example beeswax, hard paraffin or cetyl alcohol. Sweetening agents, such as those set forth below, and flavoring agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an antioxidant such as ascorbic acid. Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with
15 a dispersing or wetting agent, a suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already described above. Additional excipients, for example sweetening, flavoring and agents, may also be present.

The compounds may also be in the form of oil-in-water emulsions. The oily
20 phase may be a vegetable oil, for example olive oil or arachis oils, or a mineral oil, for example liquid paraffin or mixtures of these. Suitable emulsifying agents may be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally occurring phosphatides, for example soy bean, lecithin, and occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and
25 hexitol anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsion may also contain sweetening and flavoring agents. Syrups and elixirs may be formulated with sweetening agents, for example, glycerol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative and
30 flavoring and coloring agents.

The compounds can also be administered by injection or infusion, either subcutaneously or intravenously, or intramuscularly, or intrasternally, or intranasally, or by infusion techniques in the form of sterile injectable or oleaginous suspension. The compound may be in the form of a sterile injectable aqueous or oleaginous suspensions.

These suspensions may be formulated according to the known art using suitable dispersing of wetting agents and suspending agents which have been described above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oils may be conventionally employed including synthetic mono- or diglycerides. In addition fatty acids such as oleic acid find use in the preparation of injectables.

Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided dosages may be administered daily or the dosage may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

It is especially advantageous to formulate the compounds in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each containing a therapeutically effective quantity of the compound and at least one pharmaceutical excipient. A drug product will comprise a dosage unit form within a container that is labeled or accompanied by a label indicating the intended method of treatment, such as the treatment of an amyloid disease, such as Alzheimer's disease, or of a disease associated with alpha-synuclein fibril formation, such as Parkinson's disease. A "therapeutically effective dosage" preferably inhibits amyloidosis or a disease associated with alpha-synuclein fibril formation in a patient by at least 20, more preferably by at least 40%, even more preferably by at least 60%, and still more preferably by at least 80%, relative to untreated subjects.

Preparation of the Compounds

Many of the compounds used in disclosed methods are well known to the art and are commercially available. They may be briefly described in such references as the Merck Index, 12th edition, Merck & Co., Inc., Whitehouse Station, New Jersey, 1996 (which typically provides a reference to a synthesis or isolation), and may be found in chemical catalogs, such as those of commercial suppliers as described herein.

INDUSTRIAL APPLICABILITY

The invention finds worldwide utility in that it provides therapeutic relief and diagnostic assistance in treating and preventing Alzheimer's disease, Parkinson's disease, systemic AA amyloidosis, type 2 diabetes and other amyloidoses, by use of a
5 readily occurring and relatively inexpensive plant ingredients, or non-toxic plant derivatives, or commercially available catechins.

In compliance with the statute, the invention has been described in language
more or less specific as to structural features. It is to be understood, however, that the invention is not limited to the specific features shown, since the means and
10 construction shown comprise preferred forms of putting the invention into effect. The invention is, therefore, claimed in any of its forms or modifications within the legitimate and valid scope of the appended claims, appropriately interpreted in accordance with the doctrine of equivalents.

CLAIMS

We claim:

- 1 A method of treating an amyloid disease, or a disease characterized by alpha-synuclein or NAC fibrillogenesis, in a mammalian subject, the method comprising
5 administering to the mammal a therapeutically effective amount of a catechin selected from the group consisting of catechin, epicatechin, gallic acid gallate, epigallocatechin gallate, epigallocatechin, and epicatechin gallate, and pharmaceutically acceptable salts of the foregoing catechins.
2. The method of claim 1 wherein the selected catechin is epicatechin.
- 10 3. The method of claim 1 where the amyloid disease is selected from the group of diseases consisting of Alzheimer's disease, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis of the Dutch type, inclusion body myositis, the amyloidosis of chronic inflammation, the amyloidosis of malignancy and Familial Mediterranean Fever, the amyloidosis of multiple myeloma and B-cell dyscrasia, the
15 amyloidosis of type 2 diabetes, the amyloidosis of prion diseases, Creutzfeldt-Jakob disease, Gerstmann-Sträussler syndrome, kuru, scrapie, and mad cow disease, the amyloidosis of long-term hemodialysis, the amyloidosis of carpal tunnel syndrome, senile cardiac amyloidosis, and Familial Amyloidotic Polyneuropathy, the amyloidosis of endocrine tumors, systemic AA amyloidosis, AL amyloidosis, A β amyloidosis, IAPP
20 amyloidosis and PrP amyloidosis.
4. The method of claim 3 where the amyloid disease is Alzheimer's disease.
5. The method of claim 3 where the amyloid disease is systemic AA amyloidosis.
6. The method of claim 1 where the alpha-synuclein or NAC fibrillogenesis is a fibrillogenesis selected from the group of diseases consisting of Lewy body disease,
25 Parkinson's disease and multiple system atrophy.
7. A method of treatment of amyloid, alpha-synuclein or NAC fibrillogenesis, in an *in vitro* environment, the method comprising the step of administering into the *in vitro* environment a therapeutically effective amount of a catechin selected from the group consisting of catechin, epicatechin, gallic acid gallate, epigallocatechin
30 gallate, epigallocatechin, and epicatechin gallate, and pharmaceutically acceptable salts of the foregoing catechins.
8. The method of claim 1 further comprising an administration step whereby, the therapeutic amount of catechin is administered to the subject, selected from the group of administration steps consisting of oral administration, parenteral injection,

intraperitoneal injection, intravenous injection, subcutaneous injection, intramuscular injection, topical administration, and aerosol spray administration.

9. A pharmaceutical composition comprising a therapeutically effective amount of a catechin and a pharmaceutically acceptable carrier, diluent, or excipient, the therapeutic amount of the catechin selected for efficacy in treating amyloid, alpha-synuclein or NAC fibrillogenesis in a mammalian subject.

10. The composition of claim 8 wherein the therapeutically effective amount of the catechin comprises a dosage in the range of about 10 to 1,000 mg/kg of body weight of the subject.

11. The composition of claim 10 wherein the therapeutically effective amount of the catechin comprises a dosage in the range of about 10 to 100 mg/kg of body weight of the subject.

12. The composition of claim 9 wherein the catechin is selected from the group consisting of catechin, epicatechin, gallic acid, epigallocatechin gallate, epigallocatechin, and epicatechin gallate, and pharmaceutically acceptable salts of the foregoing catechins, and the pharmaceutically acceptable analogs and derivatives thereof.

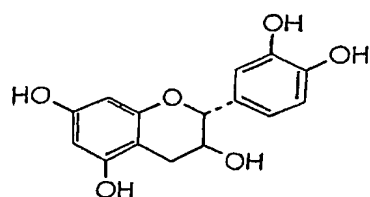
13. The composition of claim 12 comprising a mixture of two or more of the catechins selected from the group consisting of catechin, epicatechin, gallic acid, epigallocatechin gallate, epigallocatechin, and epicatechin gallate, and pharmaceutically acceptable salts of the foregoing catechins, and the pharmaceutically acceptable analogs and derivatives thereof.

14. The composition of claim 12 wherein each catechin selected is present in a percentage purity that significantly exceeds a proportion percentage of the catechin presence in a plant, or extract from a plant.

15. The composition of claim 15 wherein the catechin selected is in substantially pure isolated or synthetic form.

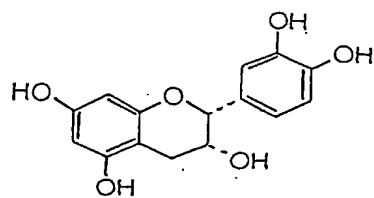
16. A method of treating an amyloid disease, or a disease characterized by alpha-synuclein or NAC fibrillogenesis, in a mammalian subject, the method comprising administering to the mammal a therapeutically effective amount of plant matter from a plant of the genus *Camellia*, species *sinensis*.

1/15



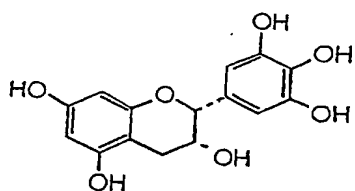
(+)-Catechin ((+)-C),

Fig. 1A



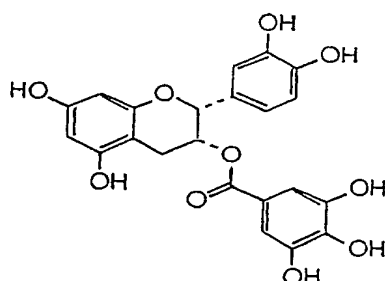
(-)-Epicatechin ((-)-EC),

Fig. 1B



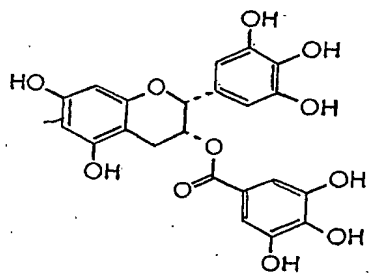
(-)-Epigallocatechin ((-)-EGC),

Fig. 1C



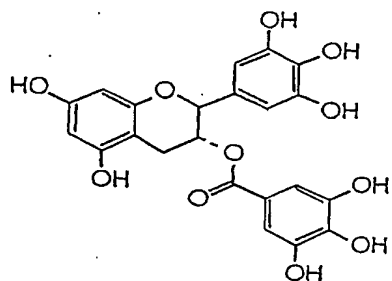
(-)-Epicatechin gallate ((-)-ECg),

Fig. 1D



(-)-Epigallocatechin gallate ((-)-EGCg),

Fig. 1E



(-)-Gallocatechin gallate ((-)-GCg)

Fig. 1F

2/15

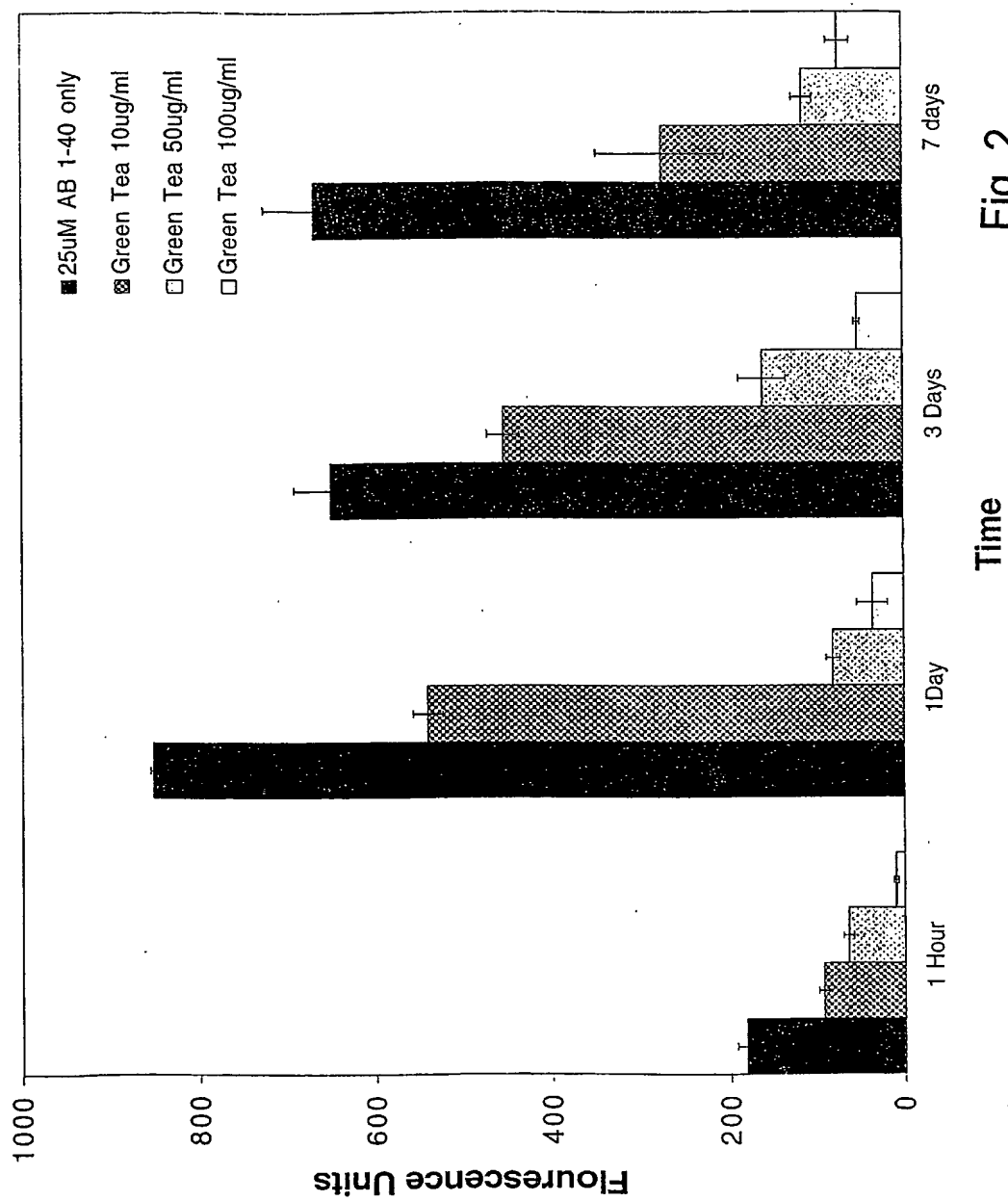


Fig. 2

3/15

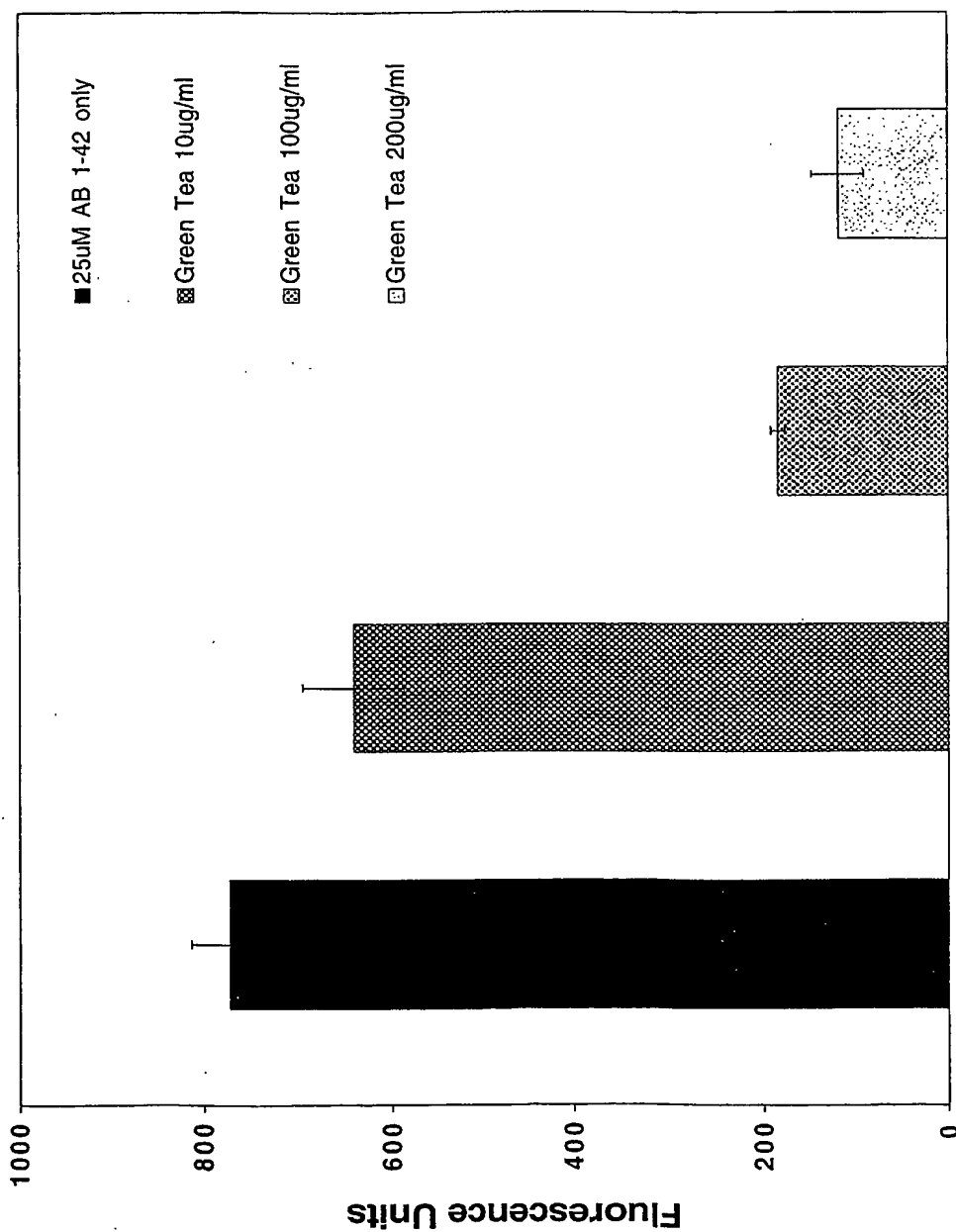


Fig. 3

4/15

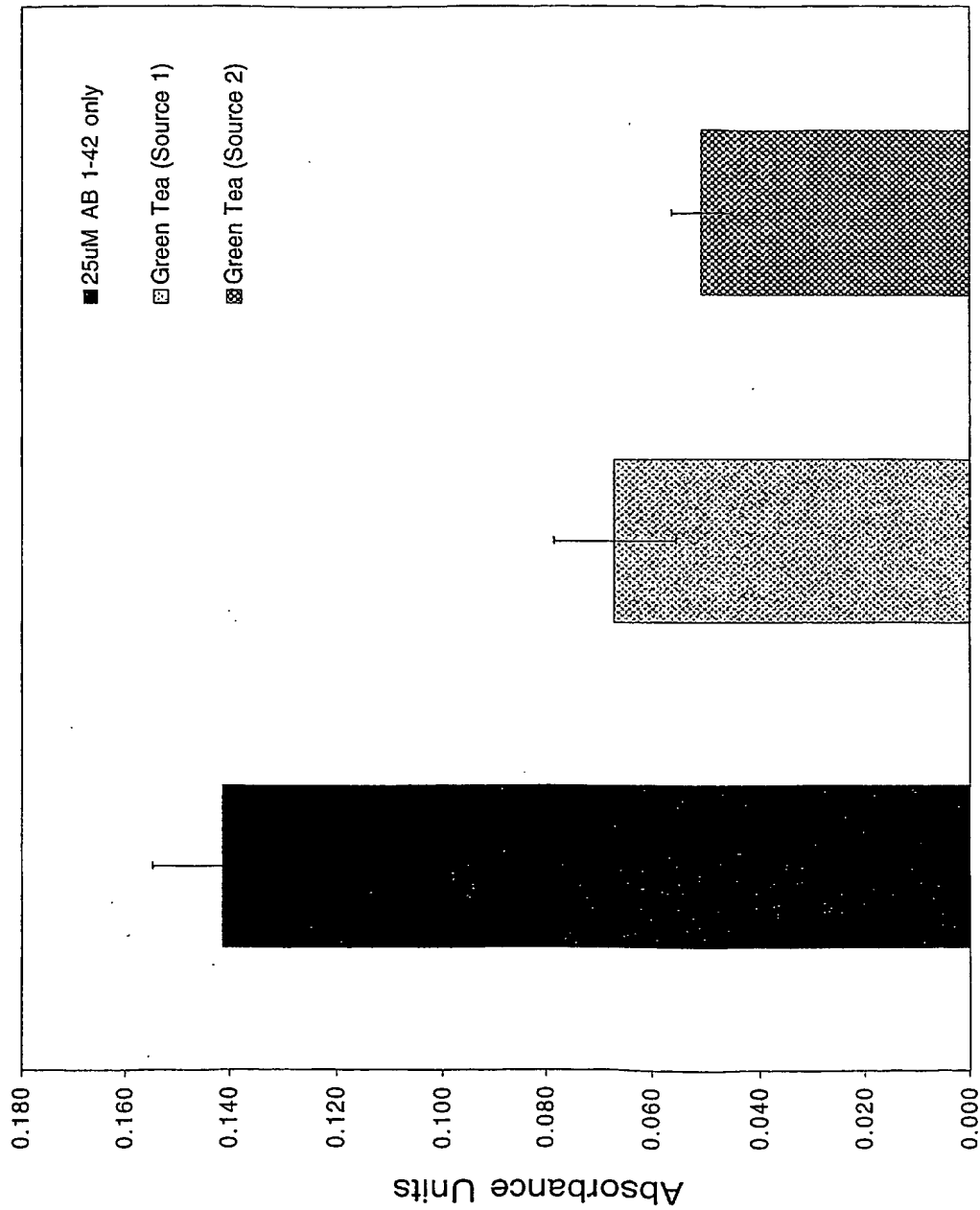


Fig. 4

Treatment

5/15

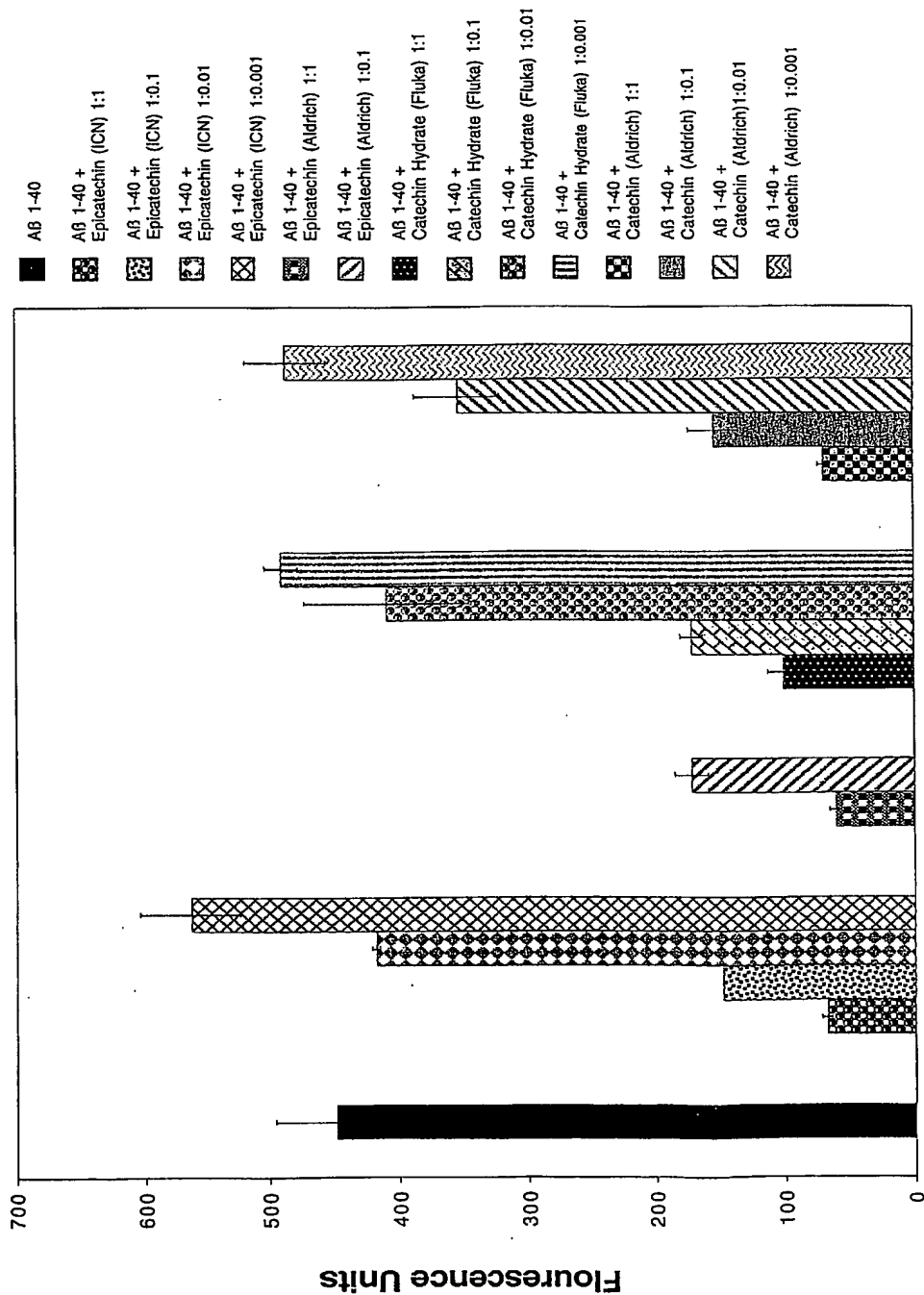


Fig. 5

Time of Treatment (7 days)

6/15

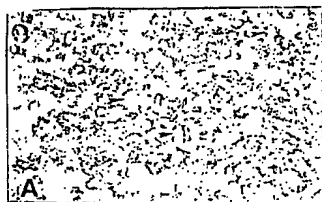


Fig. 6A

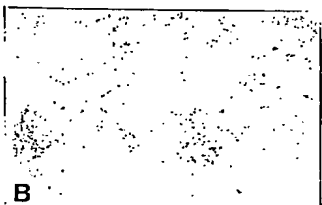


Fig. 6B



Fig. 6C

7/15

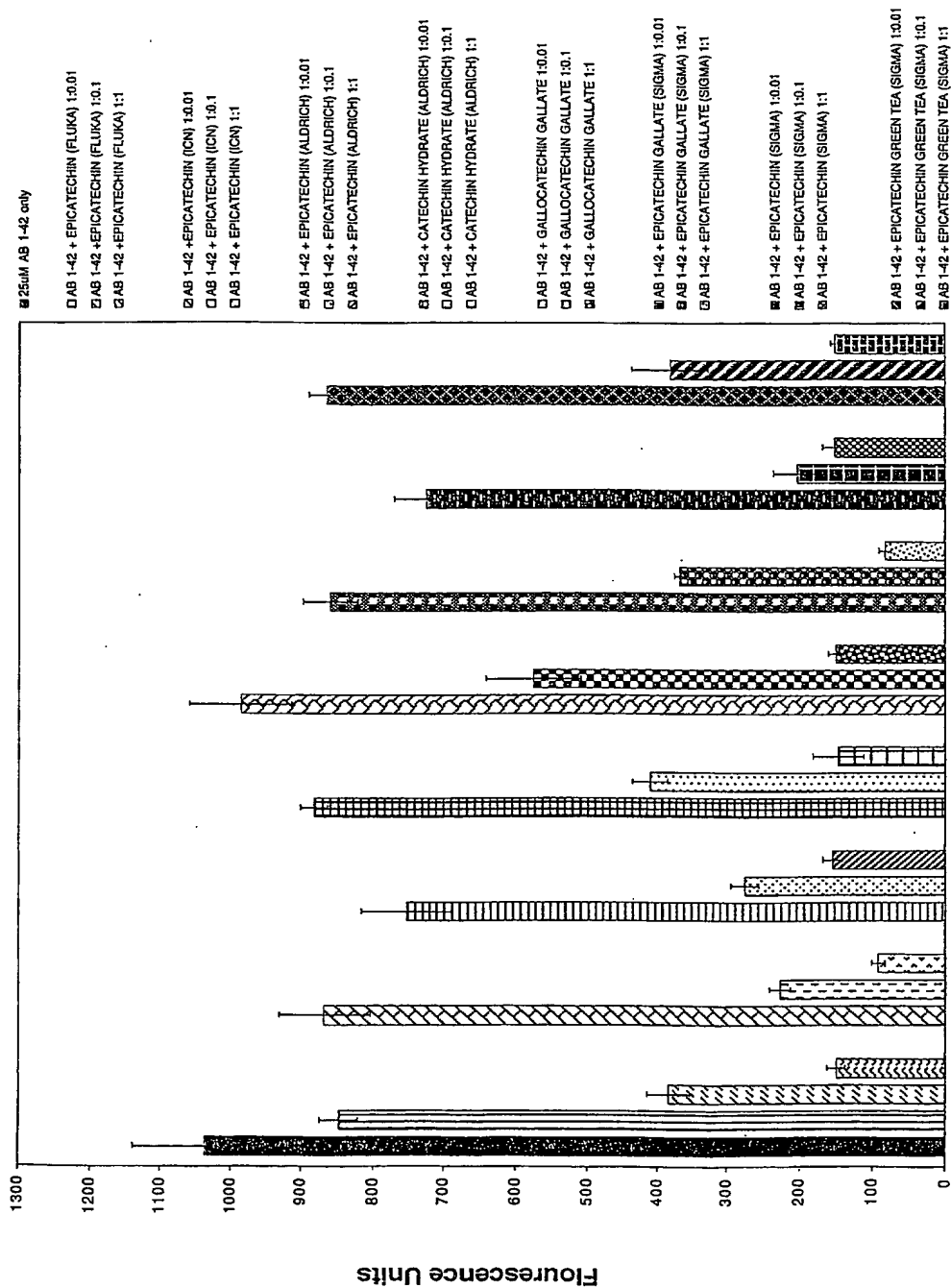


Fig. 7

Time of Treatment

8/15

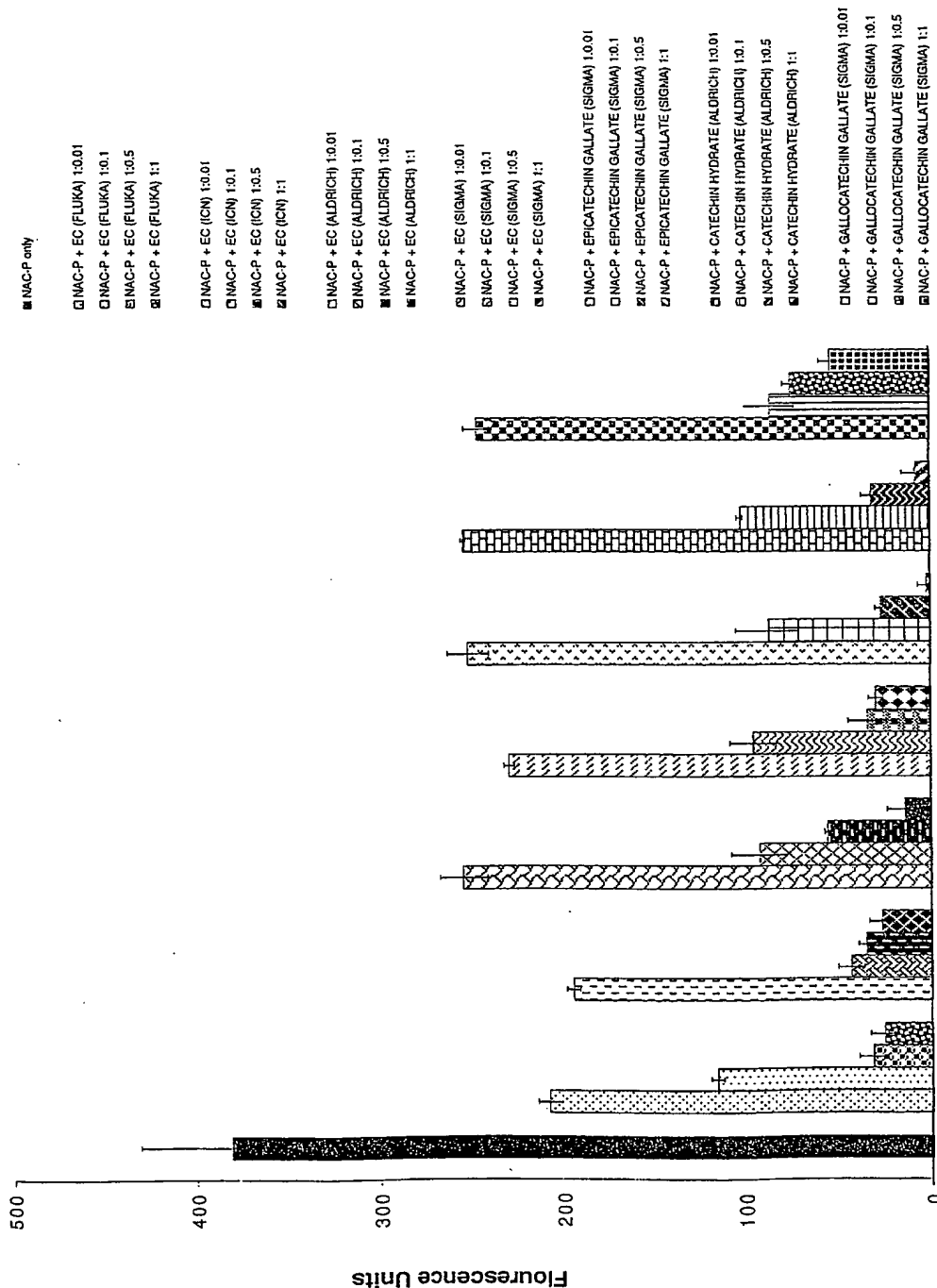


Fig. 8

Time of Treatment (7 days)

9/15

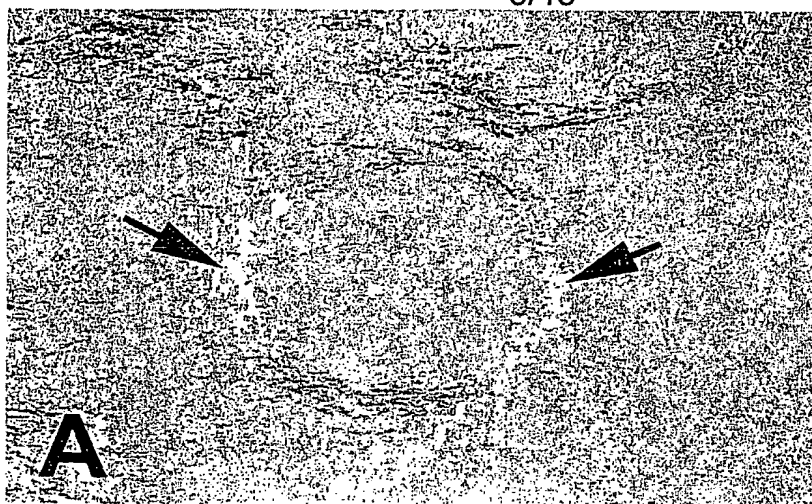


Fig. 9A

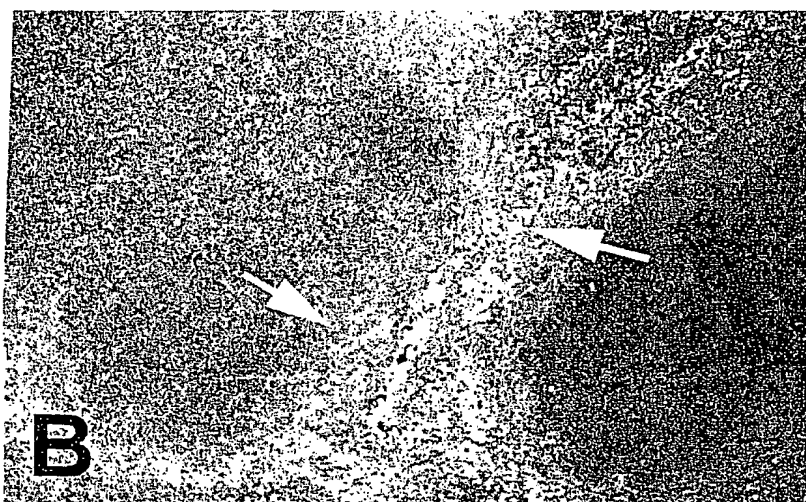


Fig. 9B



Fig. 9C

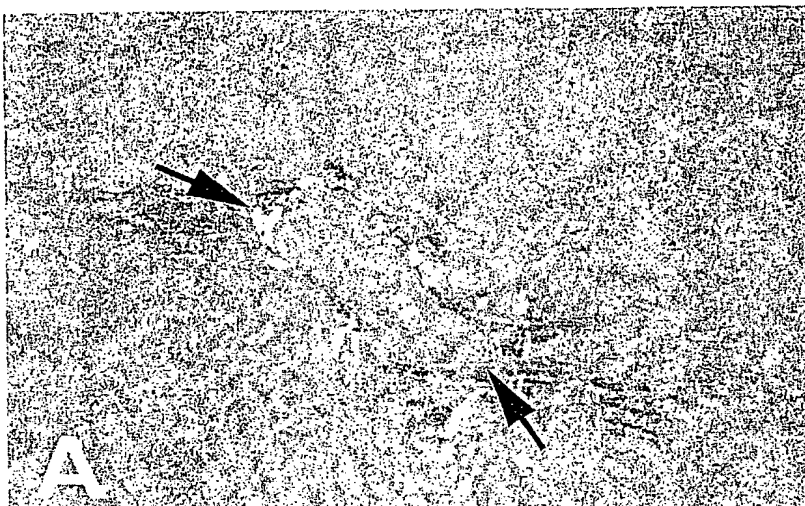


Fig. 10A

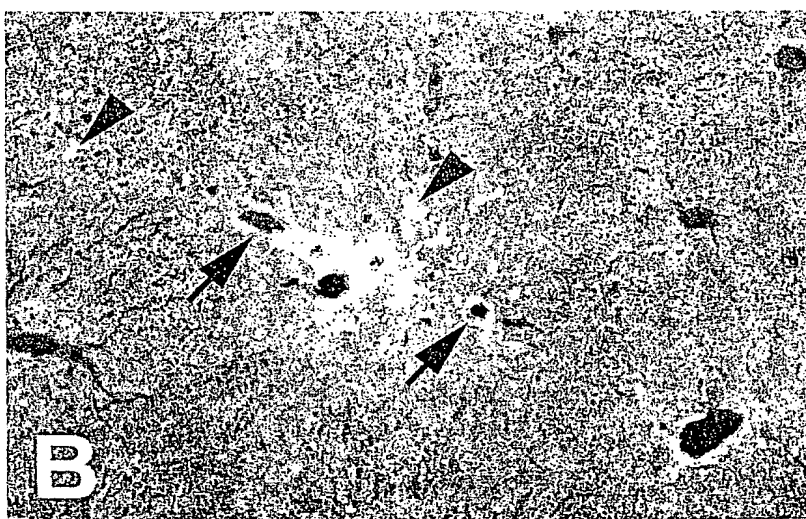


Fig. 10B

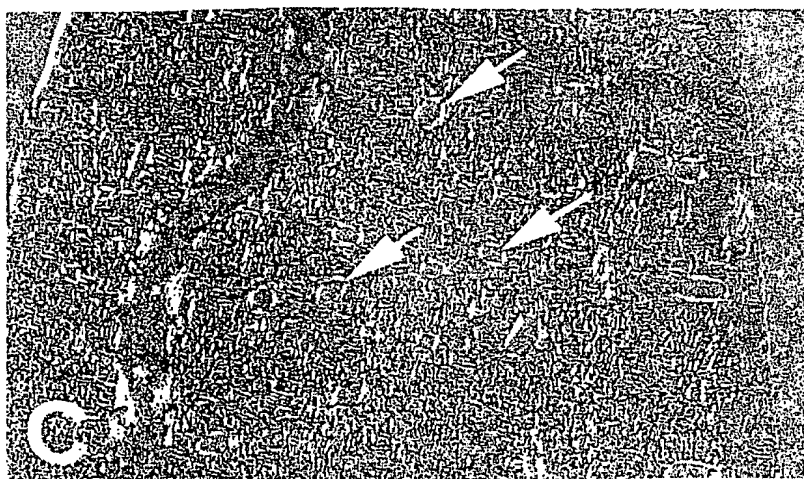


Fig. 10C

11/15

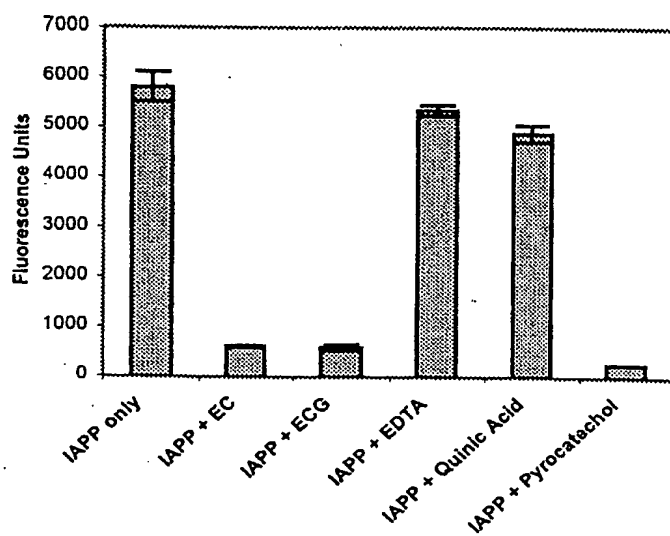


Fig. 11

12/15

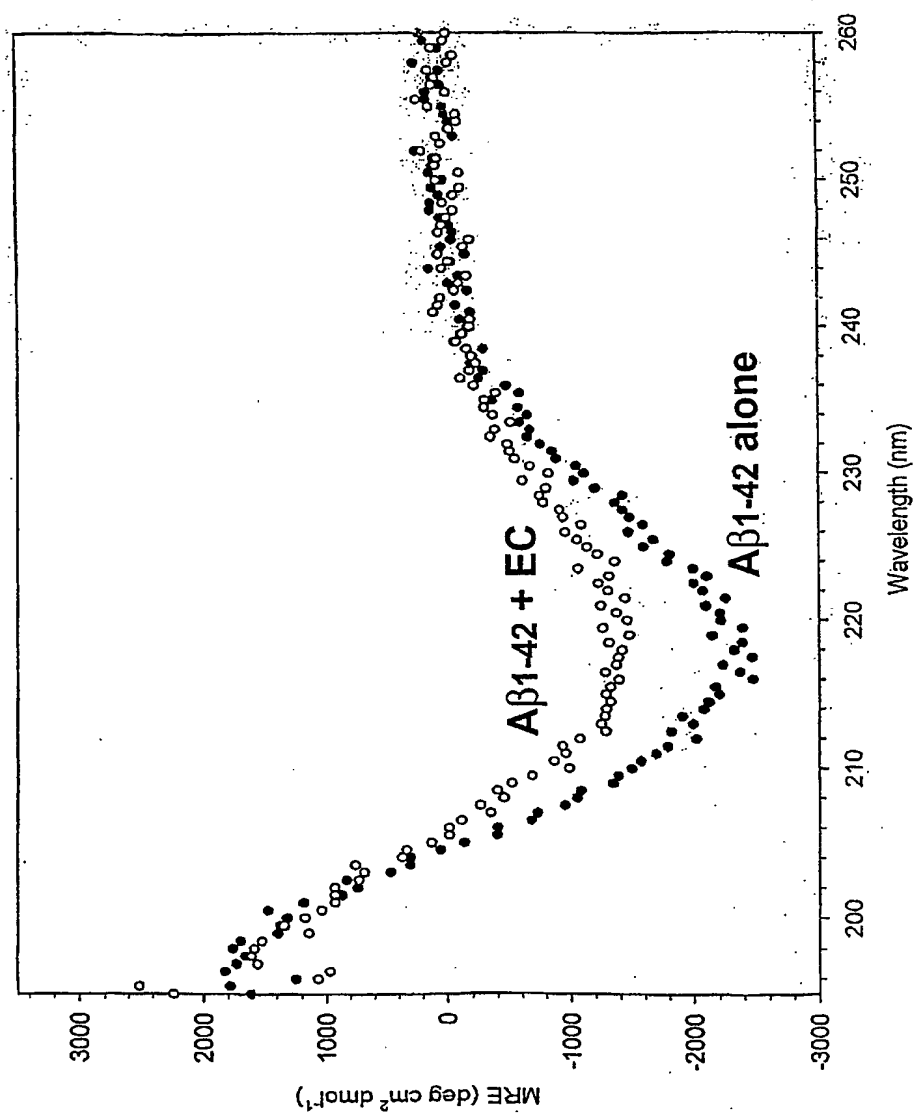


Fig. 12

13/15

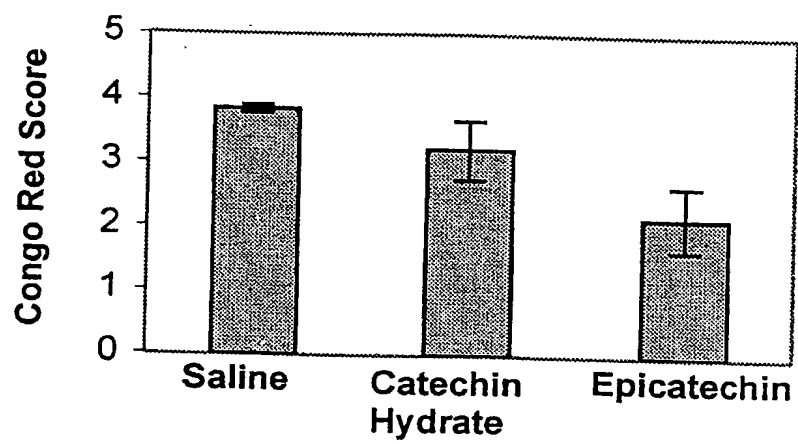


Fig. 13

14/15

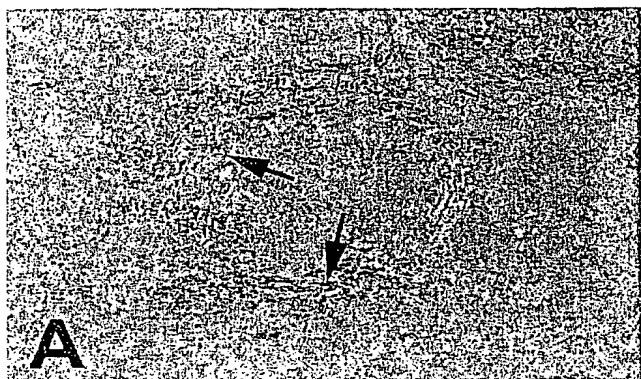


Fig. 14A

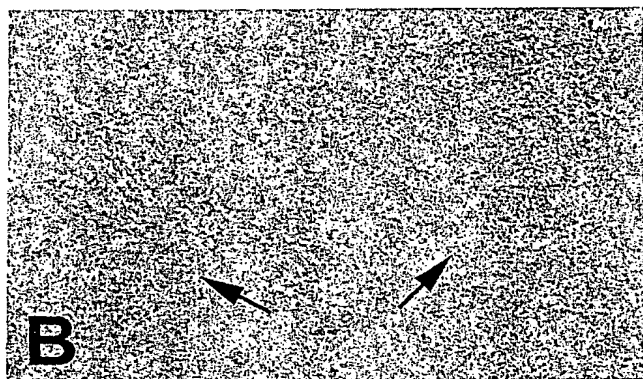
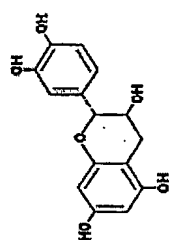


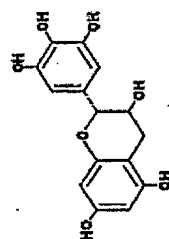
Fig. 14B

15/15



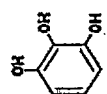
Catechin Hydrate
C₁₅H₁₄O₆

Fig. 15A



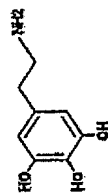
Epigallocatechin
C₁₅H₁₄O₇

Fig. 15B



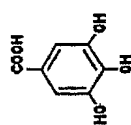
Pyrogallol
C₆H₆O₃

Fig. 15C



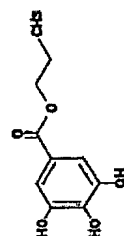
5-hydroxydopamine
C₈H₁₁NO₃

Fig. 15D



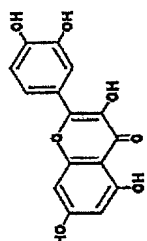
Gallic acid
C₇H₆O₅

Fig. 15E



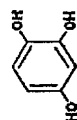
Propyl gallate
C₁₀H₁₂O₅

Fig. 15F



Quercetin
C₁₅H₁₀O₇

Fig. 15G



1,2,4-benzenetriol
C₆H₆O₃

Fig. 15H

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(88) Date of publication of the international search report:
30 May 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CATECHINS FOR THE TREATMENT OF FIBRILLOGENESIS IN ALZHEIMER'S DISEASE, PARKINSON'S DISEASE, SYSTEMIC AA AMYLOIDOSIS, AND OTHER AMYLOID DISORDERS

(57) Abstract: A method of treating an amyloid disease, or a disease characterized by alpha-synuclein or NAC fibrillogenesis, in a mammalian subject. The method includes administering to the mammal a therapeutically effective amount of a various disclosed catechins or green tea extract. A pharmaceutical composition comprising a therapeutically effective amount of a catechin and pharmaceutically acceptable excipient. The therapeutic amount of the catechin or green tea extracts is selected for efficacy in treating amyloid, alpha-synuclein or NAC fibrillogenesis in a mammalian subject.



WO 03/013442 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/07755

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 35/78, 39/385

US CL : 424/195.1; 514/451

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/195.1; 514/451

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
USPATFULL PCTFULL WPIDS MEDLINE EMABASE BIOSIS**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/51302 A1 (UNIVERSITY OF WASHINGTON) 19 November 1998 (19.11.1998), page 1-3, page 6, 9, 12, claims 1-36	1-16

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.*** Special categories of cited documents:****"A"** document defining the general state of the art which is not considered to be of particular relevance**"E"** earlier application or patent published on or after the international filing date**"L"** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)**"O"** document referring to an oral disclosure, use, exhibition or other means**"P"** document published prior to the international filing date but later than the priority date claimed**"T"**

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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document member of the same patent family

Date of the actual completion of the international search

30 May 2002 (30.05.2002)

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30 SEP 2002

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/07755

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.